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(54) Title: NITROSATED HEMOGLOBINS AND THERAPEUTIC USES THEREFOR

(57) Abstract

S-nitrosothiols (RSNOs) can donate the NO group to the β93 cysteine residues of hemoglobin (Hb) without inactivating the heme. S-nitrosylation of Hb is under the allosteric control of oxygen and the oxidation state of heme. NO group release from S-nitrosohemoglobin (SNO-Hb) is further facilitated by intracellular low molecular weight thiols, forming RSNOs which can be exported from the erythrocyte to regulate blood pressure and platelet activation. SNO-Hb can be formed by reaction of Hb with S-nitrosothiol. This procedure avoids oxidation of the heme. Other methods can be used which are not specific only for thiol groups, but which nitrosate Hb more extensively, and may produce polynitrosated metHb as a product or intermediate product of the method. SNO-Hb in its various forms and combinations thereof (oxy, deoxy, met; specifically S-nitrosylated, or nitrosated or nitrated to various extents) can be administered to an animal or human where it is desired to oxygenate, to scavenge free radicals, or to release NO^+ groups to tissues. Thiols and/or NO donating agents can also be administered to enhance the transfer of NO^+ groups. Examples of conditions to be treated by SNO-Hbs or other nitrosated or nitrated forms of Hb include ischemic injury, hypertension, angina, reperfusion injury and inflammation, and disorders characterized by thrombosis.

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NITROSATED HEMOGLOBINS AND THERAPEUTIC USES THEREFOR

RELATED APPLICATIONS

This application claims priority to U.S. Serial No. 08/667,003, filed June 20, 1996, which is a continuation-in-part of U.S. Serial No. 08/616,371, filed March 15, 1996, which claims priority to U.S. Serial No. 60/003,801, the teachings of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Interactions of hemoglobin (Hb) with small diffusable ligands, such as O_2, CO_2 and NO, are known to occur at its metal centers and amino termini. The O_2/CO_2 delivery functionalities, which arise in the lung and systemic microvasculature, are allosterically controlled. Such responsiveness to the environment is not known to apply in the case of NO. Specifically, it is thought that Hb(Fe) is involved in limiting NO's sphere of action (Lancaster, J.R., *Proc. Natl. Acad. Sci. USA, 91*:8137-8141 (1994); Wood and Garthwaite, *J. Neuropharmacol., 33*:1235-1244 (1994)), but that NO does not modify the functional properties of Hb to any physiologically significant degree. Kinetic modeling based on this assumption, however, predicts that the vast majority of free NO in the vasculature should be scavenged by Hb (Lancaster 1994). Accordingly, the steady-state level of NO may fall below the K_m for target enzymes such as guanylate cyclase (Lancaster 1994), if not in the unperturbed organism, then with oxidant stress such as that found in atherosclerosis. These considerations raise the fundamental question of how NO exerts its biological activity.

One answer to this paradox may be found in the propensity of nitric oxide to form S-nitrosothiols (RSNOs) (Gaston, B. *et al., Proc. Natl. Acad. Sci. USA, 90*:10957-10961 (1993)), which retain NO-like vasorelaxant activity (Stamler, J.S., *et al., Proc. Natl. Acad. Sci, USA, 89*:444-

448 (1992)), but which are not subject to the diffusional constraints imposed by the high concentration of Hb in the blood. In particular, the NO group of RSNOs possesses nitrosonium (NO^+) character that distinguishes it from NO itself. It is increasingly appreciated that RSNOs have the capacity to elicit certain functions that NO is incapable of (DeGroote, M.A. *et al.*, *Proc. Natl. Acad. Sci. USA, 92:*6399-6403 (1995); Stamler, J.S., *Cell, 78:*931-936 (1994)). Moreover, consideration has been given to the possibility that -SNO groups in proteins may serve a signaling function, perhaps analagous to phosphorylation (Stamler, J.S. *et al.*, *Proc. Natl. Acad. Sci. USA, 89:*444-448 (1992); Stamler, J.S. *Cell, 78:*931-926 (1994)). Although S-nitrosylation of proteins can regulate protein function (Stamler, J.S. *et al.*, *Proc. Natl. Acad Sci. USA, 89:*444-448 (1992); Stamler, J.S., *Cell, 78:*931-936 (1994)), the identification of S-nitrosoproteins within cells -- the *sine qua non* of a regulatory posttranslational modification -- has heretofore not been demonstrated.

Hemoglobin is a tetramer comprised of two alpha and two beta subunits. In human Hb, each subunit contains one heme, while the beta (β) subunits also contain highly reactive SH groups (cysβ93) (Olson, J.S., *Methods in Enzymology 76:*631-651 (1981); Antonini, E. & Brunori, M. In *Hemoglobin and Myoglobin in Their Reactions with Ligands,* American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). These cysteine residues are highly conserved among species although their function has remained elusive.

NO (nitric oxide) is a biological "messenger molecule" which decreases blood pressure and inhibits platelet function, among other functions. NO freely diffuses from endothelium to vascular smooth muscle and platelet and across neuronal synapses to evoke biological responses. Under some conditions, reactions of NO with other components present in cells and in serum can generate toxic

intermediates and products at local concentrations in tissues which are effective at inhibiting the growth of infectious organisms. Thus, it can be seen that a method of administering an effective concentration of NO or biologically active forms thereof would be beneficial in certain medical disorders.

Platelet activation is an essential component of blood coagulation and thrombotic diathesis. Activation of platelets is also seen in hematologic disorders such as sickle cell disease, in which local thrombosis is thought to be central to the painful crisis. Inhibition of platelet aggregation is therefore an important therapeutic goal in heart attacks, stroke, peripheral vascular disease and shock (disseminated intravascular coagulation). Researchers have attempted to give artificial hemoglobins to enhance oxygen delivery in all of the above disease states. However, as recently pointed out by Olsen and coworkers, administration of underivatized hemoglobin leads to platelet activation at sites of vascular injury (Olsen S.B. *et al., Circulation 93*:327-332 (1996)). This major problem has led experts to conclude that cell-free underivatized hemoglobins may pose a significant risk in the patient with vascular disease or a clotting disorder (Marcus, A.J. and J.B. Broekman, *Circulation 93*:208-209 (1996)). A new method of providing for an oxygen carrier and/or a method of inhibiting platelet activation would be of benefit to patients with vascular disease or who are otherwise at risk for thrombosis.

SUMMARY OF THE INVENTION

The invention relates to methods of forming SNO-Hb by reaction of Hb with S-nitrosothiol in procedures which avoid oxidation of the heme. The invention also includes methods of producing nitrosated (including nitrosylated at thiols or metals) and nitrated derivatives of hemoglobins

in which the heme Fe may or may not be oxidized, depending on the steps of the method. The invention also relates to a method of therapy for a condition in which it is desired to oxygenate, to scavenge free radicals, or to release NO^+ groups to tissues. SNO-Hb in its various forms and combinations thereof (oxy, deoxy, met; specifically S-nitrosylated, or nitrosated or nitrated to various extents) can be administered to an animal or human in these methods. Thiols and/or NO donating agents can also be administered to enhance the transfer of NO^+ groups. Examples of conditions to be treated by nitrosated or nitrated forms of hemoglobin include ischemic injury, hypertension, angina, reperfusion injury and inflammation, and diseases characterized by thrombosis.

BRIEF DESCRIPTIONS OF THE DRAWINGS

Figures 1A-1D are spectrographs of different forms of Hb as described in Example 1.

Figure 2A is a graph showing formation, with time, of SNO-Hb by S-nitrosylation.

Figure 2B is a graph showing the decomposition, with time, of oxy and deoxy forms of SNO-Hb.

Figure 3A is a graph showing the loading of red blood cells with S-nitrosocysteine, over time. The inset is a series of spectrographs of forms of Hb as described in Example 3.

Figure 3B is a series of tracings recording isometric tone of a rabbit aortic ring following treatment of the aortic ring with various agents as described in Example 3.

Figure 4A is a graph of change in tension of a rabbit aortic ring versus concentration of the Hb used in the experiment.

Figure 4B is a graph of change in tension of a rabbit aortic ring versus concentration of the Hb used in the

experiment, where glutathione was also added to test the effect as compared to Figure 4A.

Figure 4C is a graph of the ratio of S-nitrosoglutathione formed/starting SNO-Hb concentration versus time, showing rates of NO group transfer from oxy and met forms of Hb to glutiathione.

Figure 4D is a graph of S-nitrosothiols exported from loaded red blood cells over time.

Figure 5 is a graph showing the mean arterial blood pressure in rats after they received various doses of oxyHb (▲), SNO-oxyHb (■), or SNO-metHb (●).

Figures 6A-6F are a series of tracings recording blood pressure (Figures 6A and 6B), coronary artery diameter (Figures 6C and 6D) and coronary artery flow (Figures 6E and 6F), after administration of S-nitrosohemoglobin to anesthetized dogs.

Figure 7A is a graph illustrating the effect of unmodified HbA_0 on platelet aggregation. The maximal extent of aggregation of platelets is plotted against the concentration of HbA (10 nm to 100μm) preincubated with platelets. Experiments were performed as in Example 9. Vertical bars plotted with each data point indicate the standard deviation.

Figure 7B is a graph illustrating the effect of S-nitroso(oxy)hemoglobin on platelet aggregation. The normalized maximal extent of aggregation of platelets is plotted against the concentration of HbA (10 nm to 100μm) preincubated with platelets.

Figure 7C is a graph illustrating the antiaggregation effects on platelets by S-nitroso(met)hemoglobin.

Figure 8 is a bar graph showing the amount of cGMP (guanosine 3',5'-cyclic phosphoric acid), assayed as in Example 10, for 1, 10 and 100 μM concentrations of native Hb, SNO-oxyHb or SNO-metHb interacting with 10^8 platelets.

Figure 9A is a graph which shows the spectra (absorbance versus wavelength in nanometers) of HbA_0 treated as described in Example 11. The shift in the wavelength of maximum absorbance of spectrum B relative to spectrum A illustrates the extent of addition of NO groups to HbA_0.

Figure 9B is a graph which shows the spectra of Hb treated with 100-fold excess S-nitrosoglutathione as described in Example 11.

Figure 9C is a graph which shows the spectra of HbA_0 treated with excess S-nitrosocysteine as described in Example 11.

Figure 9D is a graph which shows the spectra of rat Hb treated with 100-fold excess S-nitrosocysteine. Spectrum A shows nitrosated Hb not further treated with dithionite; spectrum B shows nitrosated Hb further treated with dithionite.

Figure 9E is a graph illustrating the increase in nitrosated Hb product with time by reacting HbA_0 with either 100x excess S-nitrosocysteine (top curve) or 10x excess S-nitrosocysteine (middle curve). HbA_0 was preincubated with 100 μM inositol hexaphosphate before reacting with 10x excess S-nitrosocysteine (bottom curve; triangle points). (See Example 11.)

Figure 10 is a graph illustrating the percent change, with time, in blood flow measured in caudatoputamen nucleus of rats after injection of the rats with: o, 100 nmol/kg SNO-Hb; ●, 1000 nmol/kg SNO-Hb; or ■, 1000 nmol/kg underivatized Hb (see Example 12).

Figure 11 is a graph illustrating the percent change in tension of a ring of aorta from rabbit, plotted as a function of the log of the molar concentration of hemoglobin tested (see Example 13). ●, Hb treated with S-nitrosocysteine at a ratio of 1:1 CYSNO/Hb; o, Hb treated

with CYSNO at a ratio of 10:1 CYSNO/Hb; ♦, Hb treated with CYSNO at a ratio of 100:1.

DETAILED DESCRIPTION OF THE INVENTION

Roles for Hemoglobin in Physiology

The increase in SNO-Hb content of red cells across the pulmonary circuit (right ventricular inport-left ventricle) suggests that the Hb molecule is S-nitrosylated in the lung. Selective transfer of the NO group from endogenous RSNOs found in lung (Gaston, *et al.* (1993) and blood (Scharfstein, J.S. *et al.*, *J. Clin. Invest. 94*:1432-1439 (1995)) to SH groups of Hb, substantiate these findings. Nonetheless, the mechanism(s) of S-nitrosylation operative *in vivo* is not known. The corresponding decline in Hb(FeII)NO levels across the pulmonary bed reveals a role for the lung either in the elimination of NO or in its intramolecular transfer from heme to cysβ93. Taken in aggregate, these data extend the list of function-regulating interactions of Hb with small molecules within the respiratory system, previously known to include the elimination of CO and CO_2 and uptake of O_2. Since oxygenation of Hb leads to structural changes that increase the NO-related reactivity of cysβ93, O_2 may be regarded as an allosteric effector of Hb S-nitrosylation. This is a newly discovered allosteric function for the protein.

The arterial-venous difference in SNO-Hb concentration suggests that the protein acts as an NO group donor in the systemic circulation. There is good indication that SNO-Hb functions in regulation of vasomotor tone. In the microcirculation, where control of blood pressure is achieved, erythrocytes come in intimate contact with endothelial surfaces. Under these conditions, Hb can predispose the vasculature to increases in arterial

resistance by sharply decreasing the steady state level of free NO (Lancaster, J.R., (1994). This rationale is believed to contribute to the increases in blood pressure that occur with infusion of cell-free Hbs (Vogel, W.M., *et al., Am. J. Physiol., 251:*H413-H420 (1986); Olsen, S.B., *et al., Circulation 93:*329-332 (1996)). The transient nature of such hypertensive responses, however, would be consistent with the subsequent formation of SNO-Hb which counteracts this effect, evidenced by its lowering of blood pressure at naturally occurring concentrations. Thus, the capacity of the erythrocyte to support the synthesis and metabolism of SNO-Hb may well be important for normal blood flow.

It is implicit from this line of reasoning that mammals must have adopted unique molecular mechanisms to ensure adequate NO delivery in the microcirculation. Results herein suggest that Hb may have evolved both electronic and conformational switching mechanisms to achieve NO homeostasis. Specifically, NO scavenging by the metal center(s) of SNO-Hb(FeII)O_2 would be sensed through its conversion to met (FeIII) (Figure 1B). This electronic event would effect decomposition of SNO-Hb with NO group release (Figures 3A, 3B, 4A). In this manner, the NO-related activity of SNO-Hb would be partly determined by the amount of NO scavenged. Changes in O_2 tension might also function to regulate NO delivery, as it was observed that NO release was facilitated by deoxygenation. This allosteric effect may operate to limit the tissue O_2 deficit. In this scenario, NO group release would serve to regulate capillary blood flow to enhance O_2 delivery.

S-nitrosothiol groups in proteins have been implicated in NO metabolism and in regulation of cellular functions (Stamler, J.S., *et al., Proc. Natl. Acad. Sci USA, 89:*444-448 (1992); Stamler, J.S., *Cell, 78:*931-936 (1994)). The identification of SNO-Hb in erythrocytes is the first

demonstration of an intracellular S-nitrosoprotein and gives further credence to the role of such proteins in cellular regulation. The question arises as to how SNO-Hb relaxes blood vessels when any free NO released would be scavenged instantaneously by Hb itself (Lancaster, J.R., (1994)). Noteworthy in this regard are studies showing that RSNO activity involves nitrosyl (NO^+) transfer to thiol acceptors (Scharfstein, J.S., *et al.*, (1994); Arnelle, D.R. and Stamler, J.S., *Arch. Biochem. Biophys.* *318*:279-285 (1995); Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci USA*, *89*:7674-7677 (1992)), which serve to protect the NO-related activity from inactivation at metal centers. Findings presented herein indicate that S-nitrosothiol/thiol exchange with glutathione (forming GSNO) is likely to occur within erythrocytes, and to be influenced by the oxidation state of heme and its occupation by ligand. De Groote and coworkers have shown that certain activities of GSNO in bacteria require transport of intact dipeptide (i.e S-nitrosocysteinylglycine) across the cell membrane (DeGroote, M.A., *et al.*, (1995). The data presented here expand this paradigm to include eukaryotic cells. GSNO, or related thiol carriers exported by erythrocytes (Kondo, T., *et al.*, *Methods in Enzymology*, Packer, L., ed., Academic Press, *252*:72-83 (1995)), might also initiate signalling in or at the plasmalemma (Stamler, J.S., *Cell*, *78*:931-936 (1994)), given reports of thiol-dependent activation of potassium channels by EDRF (Bolotina, V.M., *et al.*, *Nature*, *368*:850-853 (1994)). Alternative possibilities also merit consideration. In particular, reports that Hb associates with red cell membranes via cysβ93 (Salhany, J.M. and Gaines, K.C.,*Trends in Biochem. Sci.*, Jan, 13-15 (1981)) would place Hb in a position to donate the NO group directly to contacting endothelial surfaces, perhaps via SNO/SH exchange. Cell surface interactions appear to be

operative in signaling mediated by other S-nitrosoproteins (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA, 89*:444-448 (1992); Stamler, J.S., *Cell, 78*:931-936 (1994)).

The highly conserved cysβ93 residues in Hb have been shown to influence the oxygenation and propensity for oxidation of the metal center, and to affect the physicochemical behavior of the protein (Garel, C., *et al.*, *Biochem. 123*:513-519 (1982); Jocelyn, P.C., *et al.*, *Biochemistry of the SH Group*, p.243, Academic Press, London; (1972); Craescu, C.T., *J. Biol. Chem. 261*:14710-14716 (1986); Mansouri, A., *Biochem. Biophys. Res. Commun., 89*:441-447 (1979)). Nonetheless, their long sought after physiological function has remained a mystery. The studies herein suggest new sensory and regulatory roles for Hb, in which cysβ93 functions in transducing NO-related signals to the vessel wall. In particular, the physiological function of cysβ93, which is invariant in all mammals and birds, is to deliver under allosteric control, NO-related biological activity that cannot be scavenged by heme. Thus, these data bring to light a dynamic circuit for the NO group in which intraerythrocytic Hb participates as both a sink and a donor, depending on the particular microenvironment of the circuit. Such observations may provide answers to paradoxes that arise from conceptual frameworks based solely on diffusional spread and reaction of free NO (Lancaster, J.R., (1994); Wood and Garthwaite, (1994)); and may have implications that extend to other thiol- and metal-containing (heme) proteins, such nitric oxide synthase and guanylate cyclase.

The discoveries reported here may have direct therapeutic implications. Specifically, concerns over loss of NO-related activity due to inactivation by blood Hb (Lancaster, J.R., (1994)) are obviated by the presence of an RSNO subject to allosteric control. SNO-Hb is free of the adverse hypertensive properties of cell-free Hb

preparations that result from NO scavenging at the metal centers. A cell-free Hb solution that mimicks blood by containing SNO-Hb can be used as a blood substitute.

Further embodiments

The subject invention relates to a method of loading cells with a nitrosating agent as exemplified for red blood cells as in Figure 3A, but which can be accomplished in more general ways. Suitable conditions for pH and for the temperature of incubation are, for example, a range of pH 7-9, with pH 8 being preferred, and a temperature range of 25 to 37°C. For red blood cells, short incubation times of 1 to 3 minutes are preferred for limiting the formation of S-nitrosylated forms of Hb. However, intracellular concentrations of 1 mM nitrosating agent can be reached.

The nitrosating agent should be a good donor of NO^+ and should be able to diffuse through the cell membrane of the target cell type. That is, it must be of low molecular weight, in contrast to S-nitrosoproteins. Examples are S-nitroso-N-acetylcysteine, S-nitrosocysteinylglycine, S-nitrosocysteine, S-nitrosohomocysteine, organic nitrates and nitrites, metal nitrosyl complexes and other related nitrosating agents as defined in "Donors of Nitrogen Oxides" pp. 71-119 In *Methods in Nitric Oxide Research* (Freelisch, M. and Stamler, J.S., eds.) Wiley, Chichester, U.K. (1996), the contents of which chapter are hereby incorporated by reference in their entirety. Nitrosating agents have differential activities for different reactive groups on metal-containing proteins. A nitrosating agent can be chosen for minimal oxidation of the heme iron of Hb, and maximum activity in nitosylating thiol groups such as found on cysteine.

Such low molecular weight nitrosating agents can be used in red blood cells to deliver NO-related activity to

tissues. Treatment of red blood cells with nitrosating agent further serves to increase the O_2 delivery capacity of red blood cells. Such treatment of red blood cells also allows for the scavenging of oxygen free radicals throughout the circulation. Therefore, it is possible to load red blood cells with S-nitrosothiol, for example, by a process outside a patient's body after removal of whole blood (as a minimal method of isolating the red blood cells) and then to reintroduce the red blood cells into the same patient), thereby allowing the treatment of a number of types of diseases and medical disorders, such as those which are characterized by abnormal O_2 metabolism of tissues, oxygen-related toxicity, abnormal vascular tone, abnormal red blood cell adhesion, or abnormal O_2 delivery by red blood cells. Such diseases can include, but are not limited, to ischemic injury, hypertension, shock, angina, stroke, reperfusion injury, acute lung injury, sickle cell anemia, schistosomiasis and malaria. The use of such "loaded" red blood cells also extends to blood substitute therapy and the preservation of living organs, as organs for transplantation, for example. In some cases, it may be appropriate to treat a patient with loaded red blood cells originating from a different person.

A particular illustration of the mechanism of the treatment method is presented here by considering sickle cell anemia. Sickle cell patients suffer from frequent vascular occlusive crises which manifest in clinical syndromes such as the acute chest syndrome and hepatic dysfunction. Both endothelial cell dysfunction, resulting in a clotting diathesis as well as dysfunction intrinsic to the red blood cell, are central to disease pathogenesis. At the molecular level, the increased expression of vascular adhesion molecules such as VCAM promote the adhesion of sickled red blood cells containing abnormal hemoglobin. It follows that decreasing cytokine expression

on endothelial cells, promoting endothelial function and attenuating red cell sickling, are key therapeutic objectives. However, currently used therapies have been generally unsuccessful.

In this novel method for loading red blood cells with intracellular NO-donor S-nitrosothiols, the effect is to increase oxygen affinity -- which in and of itself should attenuate red blood cell sickling -- and to endow the red blood cell with vasodilator and antiplatelet activity, which should reverse the vasoocclusive crisis. Moreover, nitric oxide should attenuate the expression of adhesion molecules on endothelial cell surfaces, thus restoring endothelial function.

Herein is described a novel therapeutic approach to the treatment of sickle cell disease which involves loading of red blood cells with S-nitrosothiols or other nitrosating agents. Two examples of therapeutic approaches are given. In the first, the patient's own red blood cells are S-nitrosylated extracorporeally (yielding "loaded" red blood cells) and then given to the patient. The second approach is to directly administer to a patient an agent such as S-nitrosocysteine, which is permeable to red blood cells.

For some diseases or disorders, the administration of NO-loaded red blood cells is especially desirable. Upon a change from the oxygenated to the deoxygenated state, or upon a change in the oxidation state of the heme Fe from the reduced state (FeII) to the oxidized (FeIII) state, NO is released from the thiol groups of hemoglobin, and is rapidly transferred to glutathione to form S-nitrosoglutathione. Red blood cells are known to have a high concentration of glutathione. S-nitrosoglutathione efficiently delivers NO to tissues.

In another aspect, the invention is a method for the treatment of infection by administering to an infected

mammal an agent which causes S-nitrosylation of thiol groups within the cells which are the target of such agent. For example, an S-nitrosothiol to which lymphocytes are highly permeable can be administered to a patient infected with HIV. Such treatment for HIV can also be used excorporeally, to blood isolated from the patient. In another application, the infection is bacterial, and the S-nitrosothiol to be used as an anti-bacterial agent is one to which the target bacterial cells are highly permeable, as compared to the permeability properties of the host cells. (See, for example De Groote, M.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 92:6399-6403 (1995).) Alternatively, nitrosothiols can be used to treat *Plasmodium falciparum* within red blood cells.

Another embodiment of the invention is a method for specifically modifying a protein containing one or more metal atoms so that the protein becomes S-nitrosylated at one or more thiol groups without modifying the metal, as by changing the oxidation state or causing the metal atoms to bind NO. This can be accomplished by the use of a reagent which possesses NO^+ character, such as a nitrosothiol (See, for instance, Example 4A.), which reacts specifically with thiol groups of a protein in which metal is bound.

For hemoglobin, the nitrosation method does not affect the heme. SNO-Hb (SNO-Hb(FeII)O_2) can be synthesized from Hb(FeII)O_2 with up to 2 SNO groups per tetramer without oxidation of the heme Fe from FeII to FeIII. In contrast, when Hb(FeII)O_2 is incubated with excess nitric oxide or nitrite, methemoglobin (HbFe[III]) forms rapidly (Example 1B) and to a significant extent. When Hb[FeII] is incubated with nitric oxide, NO binds rapidly to the heme, forming Hb(FeII)NO to a significant extent (Example 1A).

Although rates of formation of SNO-Hb(FeII)O_2 from Hb(FeII)O_2 are more rapid (see Example 2A), the corresponding SNO-deoxyHb form can also be made by

incubation of S-nitrosoglutathione or S-nitrosocysteine, for example, with Hb(FeII), yielding SNO-Hb(FeII), as in Example 1C.

The effects of the various forms of Hb on vasodilation -- constriction, dilation or a neutral effect -- depend on three factors: whether 1) the Fe of the heme is oxidized, 2) O_2 is bound at the heme (that is, the oxygenation state, dictated by the conformation of the protein as R state or T state), and 3) thiol is present in sufficient concentration to facilitate the transfer of NO•.

The importance of the first factor is shown in Figure 4A. Hb(FeII)O_2 and SNO-Hb[FeII]O_2 act as vasoconstrictors, but SNO-Hb[FeIII] (met form, where FeII has been oxidized to FeIII) acts as a vasodilator. Figure 4A shows that SNO-Hb[FeII]O_2 with oxygen bound at the heme, and with a ratio of SNO/Hb=2, acts as a powerful vasoconstrictor.

SNO-Hb(FeII) is also a vasodilator. Figure 2B illustrates the second factor in demonstrating that rates of RSNO decomposition and transfer are much faster for SNO-Hb in the deoxy state than for SNO-Hb in the oxy state.

It can be seen how the NO•-donating properties of SNO-Hb depend on oxygen concentrations. SNO-Hb releases oxygen at sites of low oxygen concentration or under oxidizing conditions. SNO-Hb releases its NO group(s) to cause vasodilation either due to 1) oxidation of the heme Fe to FeIII or 2) loss of the O_2 from the heme by deoxygenation. It is shown in Figure 2B that NO is transferred off SNO-Hb best in the deoxy state. In ischemia, SNO-Hb deoxygenates, rapidly followed by the loss of NO. It can be seen from the data that SNO-metHb having a ratio of 1 SNO/SNO-metHb is a more powerful vasodilator than SNO-oxyHb having a ratio of 2 SNO/SNO-oxyHb. It should be noted that S-nitrosylation of Hb induces the R state (oxy conformation). Thus, it follows that 1 SNO-oxyHb molecule having a ratio

of 1 SNO/SNO-oxyHb is less potent than 10 molecules of SNO-oxyHb having a ratio of 0.1 SNO/SNO-oxyHb.

The third factor is illustrated by the results shown in Figure 4B. These results demonstrate potentiation by thiol of the vasodilator effect of SNO-Hb(FeII)O_2 and SNO-Hb(FeIII). Transfer of NO^+ from SNO-Hb to low molecular weight nitrosothiols is more efficient when Hb is in the deoxy state compared to the oxy state (Figure 2B) or in the met state compared to the oxy state (Figure 4C).

NO is released or transferred as NO^+ (nitrosyl cation) from SNO-Hb. The SNO groups of SNO-Hb have NO^+ character. Transfer of NO^+ from SNO-Hb occurs most efficiently to small thiols, such as glutathione, and is most efficient when the heme is oxidized (SNO-metHb) or the SNO-Hb is in the deoxy state.

One embodiment of the invention resulting from these findings is a method of therapy that enhances the transfer of NO^+ from SNO-Hb to small thiols, thereby delivering NO biological activity to tissues, by the coadminstration of small molecular weight thiols, along with a form of SNO-Hb, to a mammal in need of the physiological effects of NO. To further increase the effect of NO release it is preferred that the SNO- forms of metHb or deoxyHb (or an equivalent conformation or spin state) be administered with the thiol (See Figure 2B, for example.) A mixture of SNO-metHb and SNO-oxyHb, and possibly also thiol, can also be used. The composition and proportion of these components depends on the disease state. For example, to achieve both enhanced O_2 delivery and NO delivery, SNO-oxyHb can be used. Where no further delivery of O_2 is desirable, as in acute respiratory distress syndrome, for example, the SNO- forms of metHb and deoxyHb are especially preferred. Alternatively, the ratios of SNO/Hb can be regulated to control O_2 release.

The vessel ring bioassay data of Figure 4A agree well with the *in vivo* data of Figure 5. The results of the experiments described in Example 5 confirm that Hb(FeII)O_2 (oxyHb) causes an increase in blood pressure *in vivo*, as it did also *in vitro*. SNO-Hb(FeIII) (SNO-metHb) causes a decrease in blood pressure *in vivo* as well as *in vitro*. SNO-Hb(FeII)O_2 (SNO-oxyHb) has a negligible effect on blood pressure *in vivo* in contrast to the increase in tension seen in the corresponding vessel ring bioassay. For SNO-oxyHb the *in vivo* effect is neutral. This may be explained by the constrictive effect caused by NO becoming bound to the heme being compensated by the release of NO upon deoxygenation. Therefore, SNO-oxyHb can deliver O_2 with minimal effect on blood pressure.

With knowledge of the results herein it is possible to synthesize Hb proteins with predicted NO-releasing properties, which will constrict, dilate, or have no effect on blood vessels. An additional option is the choice between making oxygenated or deoxygenated forms to administer for medical conditions in which O_2 delivery is desirable, or undesirable, respectively.

It is possible to produce a variety of modified Hbs having specific desired properties of O_2 and NO delivery. For example, Hb in the R state or R-structure (oxyHb) can be converted to the T state or T-structure (deoxyHb) by a number of known methods. This can be done, for example, by reaction of Hb with inositol hexaphosphate. It is also known to those skilled in the art that Hb in the R state can be made, for example, by treating Hb with carboxypeptidase. Similarly, it is known that metHb can be synthesized using ferricyanide or nitrite.

Producing Hb molecules which are locked in the T state allows the synthesis of RSNO-Hb which remains in a form that is a biologically active donor of NO, rather than a carrier of NO. Hb which is locked in the R state can be

used as a substrate for the synthesis of RSNO-Hb which carries a maximum amount of NO per molecule.

Another embodiment of the invention is a blood substitute comprising one or more forms of Hb which have been specifically S-nitrosylated to some extent at one or more thiol groups of the Hb, in order to regulate O_2 release and NO release. Conditions to be treated include those in which NO or O_2 delivery is desired, those in which NO or O_2 utilization is desired, or those in which NO or O_2 is in excess. For example, in a medical condition which is characterized by the presence of an excess of oxygen free radicals and excess NO·, both the heme of SNO-Hb and NO released by SNO-Hb serve to trap oxygen free radicals. The heme Fe is oxidized in the process of scavenging oxygen free radicals and NO·, and NO is released from the oxidized Hb by donation to a thiol, in the form of RSNO·, which is not toxic. Inflammation and reperfusion injury, for example, are characterized by excess NO production and an excess of oxygen free radicals. Forms of Hb scavenge oxygen radicals and free NO, converting NO to forms that are not toxic.

A further embodiment of the invention is a method of therapy for a condition that would benefit from the delivery of NO in a biologically active form or O_2 or both, based on the administration of a blood substitute comprising a form of nitrosated Hb. For example, SNO-Hb is useful to treat myocardial infarction. SNO-Hb has the effect of donating NO, keeping blood vessels open. SNO-Hb deoxygenates at low oxygen tension, delivering oxygen and releasing NO at the same site, thereby causing vasodilation. (See Example 7 and Figures 6A-6F.) These effects can be augmented by also administering thiol, either simultaneously with SNO-Hb, or before or after. For the purpose of treating myocardial infarction, for example, a high concentration or dose of SNO-Hb that has a low ratio

of SNO/SNO-Hb is appropriate. Alternatively, SNO-metHb can be used for this purpose.

In another aspect, the invention is a method of enhancing NO-donor therapy by coadministering SNO-Hb or other forms of nitrosated Hb together with a nitroso-vasodilator (nitroglycerin, for example) which would be otherwise consumed by the conversion of oxyHb to metHb in Hb which has not been S-nitrosated.

Platelet activation is manifested by a number of events and reactions which occur in response to adhesion of platelets to a nonplatelet surface such as subendothelium. Binding of agonists such as thrombin, epinephrine, or collagen sets in motion a chain of events which hydrolyzes membrane phospholipids, inhibits adenylate cyclase, mobilizes intracellular calcium, and phosphorylates critical intracellular proteins. Following activation, platelets secrete their granule contents into plasma, which then allow the linking of adjacent platelets into a hemostatic plug. (See pages 348-351 in *Harrison's Principles of Internal Medicine*, 12th edition, eds. J.D. Wilson *et al.*, McGraw-Hill, Inc., New York, 1991).

A thrombus is a pathological clot of blood formed within a blood vessel or the heart. It may remain attached to its place of origin or become dislodged and move to a new site within the circulatory system. Thromboembolism occurs when a dislodged thrombus or part of a thrombus partially or completely occludes a blood vessel and prevents oxygen transport to the affected tissues, ultimately resulting in tissue necrosis.

Sites where damage has occurred to the vascular surface are especially susceptible to the formation of thrombi. These sites include those on the interior surface of a blood vessel in which damage to the endothelium, narrowing or stenosis of the vessel, or atherosclerotic plaque accumulation has occurred.

NO is one of several endothelium-derived thromboregulators, which are defined as physiological substances that modulate the early phases of thrombus formation. In particular, NO reduces platelet adhesion, activation and recruitment on the endothelial cell surface, and achieves this, it is thought, by activating platelet guanylate cyclase, thereby increasing platelet intracellular cGMP (Stamler, J.S. *et al, Circ. Res. 65*:789-795 (1989)), and decreasing intraplatelet Ca^{2+} levels. NO and the prostacylcin prostaglandin (PG) I_2 act synergistically to inhibit and actively mediate platelet disaggregation from the collagen fibers of the subendothelial matrix. Unlike prostacyclin, NO also inhibits platelet adhesion. Furthermore, platelets synthesize NO, and the L-arginine-NO pathway acts as an intrinsic negative feedback mechanism to regulate platelet reactivity. NO is involved in leukocyte interactions with the vessel wall and can inhibit neutrophil aggregation. (See review article, Davies, M.G. *et al., British Journal of Surgery 82*:1598-1610, 1995.)

NO is antiathrogenic in a number of ways. (See, for example, Candipan, R.C. *et al., Arterioscler. Thromb. Vasc. Biol. 16*:44-50, 1996.) NO inhibits smooth muscle proliferation and attenuates LDL (low density lipoprotein) oxidation and other oxidant-related processes.

Hemoglobin may promote atherosclerosis as well as thrombosis as a consequence of its NO-scavenging property. This limitation of hemoglobin derives from its high affinity for nitric oxide. *In vitro*, NO is a potent inhibitor of platelet aggregation and adhesion to collagen fibrils, the endothelial cell matrix and monolayers (Radomski, M.W. *et al., Br. J. Pharmacol. 92*:181-187 (1987); Radomski, M.W. *et al., Lancet 2*:1057-1058 (1987); Radomski M.W. *et al., Biochem. Biophys. Res. Commun. 148*:1482-1489 (1987)). NO elevates cGMP levels in

platelets, thereby decreasing the number of platelet-bound fibrinogen molecules and inhibiting intracellular Ca^{++} flux and platelet secretion (Mellion, B.T. *et al.*, *Blood* 57:946-955 (1981); Mendelson, M.E. *et al.*, *J. Biol. Chem.* 165:19028-19034 (1990); Lieberman, E. *et al.*, *Circ. Res.* 68:1722-1728 (1991)). Scavenging of nitric oxide by Hb prevents the molecule from inhibiting platelets. This explanation has been given support by *in vivo* studies (Krejcy, K. *et al.*, *Arterioscler. Thromb. Vasc. Biol.* 15:2063-2067 (1995)).

The results shown in Figure 7A-7C (see Example 9) show that nitrosated hemoglobins, including SNO-Hb, can be used in the treatment of acute blood clotting events that occur as a result of increased platelet deposition, activation and thrombus formation or consumption of platelets and coagulation proteins. Such complications are known to those of skill in the art, and include, but are not limited to myocardial infarction, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis and unstable angina, and any additional complication which occurs either directly or indirectly as a result of the foregoing disorders.

SNO-Hb and other nitrosated hemogobins can also be used prophylactically, for example, to prevent the incidence of thrombi in patients at risk for recurrent thrombosis, such as those patients with a personal history or family history of thrombosis, with atherosclerotic vascular disease, with chronic congestive heart failure, with malignancy, or patients who are pregnant or who are immobilized following surgery.

NO is known to activate soluble guanylate cyclase, which produces cGMP. cGMP mediates inhibition of platelet aggregation. Results in Example 10 demonstrate that this inhibition of platelet aggregation may be mediated not by cGMP alone, but by some other mechanism as well.

Certain compounds or conditions are known to cause a shift in the allosteric equilibrium transition of Hb towards either of the two alternative quaternary structures of the tetramer, the T- or R-structures. (See, for example, pages 7-28 in Perutz, M., *Mechanisms of Cooperativity and Allosteric Regulation in Proteins*, Cambridge University Press, Cambridge, U.K., 1990.) These are, for instance, the heterotropic ligands H^+, CO_2, 2,3-diphosphoglycerate (2,3-DPG) and Cl^-, the concentrations of which modulate oxygen affinity. The heterotropic ligands lower the oxygen affinity by forming additional hydrogen bonds that specifically stabilize and constrain the T-structure. Other compounds affecting the allosteric equilibrium include inositol hexaphosphate (IHP) and the fibric acid derivatives such as bezafibrate and clofibrate. The fibric acid derivatives, antilipidemic drugs, have been found to combine with deoxy-, but not with oxyhemoglobin. They stabilize the T-structure by combining with sites in the central cavity that are different from the DPG binding sites. Other allosteric effectors have been synthesized which are related to bezafibrate. A ligand that stabilizes specifically the R-structure increases the oxygen affinity, and a ligand that stabilizes the T-structure does the reverse. Other ligands may affect the spin state of the heme. For example, in deoxyhemoglobin and in methemoglobin the Fe is high-spin ferrous (S=2) and 5-coordinated; in oxyhemoglobin and in cyan-metHb the Fe is low-spin ferrous (S=0) and 6-coordinated; when H_2O is the sixth ligand, methemoglobin is also high-spin. The inhibition of platelet aggregation by S-nitroso-methemoglobin seen in Figure 7C is consistent with enhanced potency in the high spin conformation. Such substances which control the allosteric equilibrium or spin state of hemoglobin may be administered to a human or animal to promote the formation

of, or to stabilize, a particular allosteric structure and/or spin state.

The dosage of Hb required to deliver NO for the purpose of platelet inhibition can be titrated to provide effective amounts of NO without causing drastic changes in blood pressure. If the goal of the therapy is to deliver oxygen, the Hb may be administered in a unit of blood to avoid a drop in blood pressure. If the goal is to alleviate shock, very little Hb can be administered compared to the amount to be given for myocardial infarction. For shock, the more important goal is to deliver NO rather than to deliver oxygen. It may be preferable to use continuous infusion or several infusions per day. Example 12 (see Figure 10) shows that the effects of SNO-Hb(FeII)O_2 on blood flow in rat brain last over 20 minutes; in other experiments an effect has been seen for up to an hour. There is a correlation between blood pressure effects and platelet inhibition effects, but platelet inhibition occurs at a lower NO concentration than that which is required to produce blood pressure effects, and generally lasts longer.

Example 11 shows that S-nitrosothiols can be used to add NO groups not only on the thiol groups of cysteine residues in hemoglobin, but also on other reactive sites of the hemoglobin molecule. The products of the nitrosation reactions in Example 11 were hemoglobin molecules with more than 2 NO groups per Hb tetramer. The exact sites of the addition of NO have not been confirmed, but it is expected that NO addition occurs at thiol groups and various other nucleophilic sites within Hb, including metals. Reactive sites, after the thiol groups, are tyrosine residues and amines, and other nucleophilic centers.

Nitrosation reactions on other proteins have been investigated previously (Simon, D.I. *et al.*, *Proc. Natl. Acad. Sci. USA* 93:4736-4741 (1996)). Methods of modifying

proteins to produce nitrosoproteins are known in the art, and include, for example, exposing the protein to $NaNO_2$ in 0.5 M HCl (acidified NO_2^-) for 15 minutes at 37°C. An alternative method is to place a helium-deoxygenated solution of protein in 100 mM sodium phosphate, pH 7.4, inside dialysis tubing and expose the protein to NO gas bubbled into the dialysate for 15 minutes. (Stamler, J.S. *et al.*, *Proc. Natl. Acad. Sci. USA 89*:444-448 (1992); see also Williams, D.L.H. *Nitrosation*, Cambridge University Press, New York (1988), which gives further methods of nitrosation).

By these methods, multiple NO-related modifications ("NO groups" or "NO biological equivalents" resulting from nitrosations, nitrosylations or nitrations) can be made on Hb at nucleophilic sites, which may include thiols, nucleophilic oxygen atoms as may be found in alcohols, nucleophilic nitrogen atoms as may be found in amines, or the heme iron. Agents facilitating nitrosations, nitrosylations or nitrations of Hb can be thought of as "NO or NO^+ donating agents." The products of such modifications may have such groups, for example, as -SNO, -SNO_2, -ONO, ONO_2, -CNO, -CNO_2, -NNO, -NNO_2, -FeNO, -CuNO, -SCuNO, SFeNO and the different ionized forms and oxidation variants thereof. (See, regarding oxidation of hemoglobin by Cu^{++}, Winterbourne, C., *Biochemistry J. 165*:141-148 (1977)). The covalent attachment of the NO group to sulfydryl residues in proteins is defined as S-nitrosylation; the covalent attachment of the NO group to a metal, such as Fe, can be called nitrosylation. General NO attachment to nucleophilic centers is referred to as nitrosation. Thus, the term nitrosated hemoglobin includes SNO-Hb and Hb(FeII)NO as well as other forms of hemoglobin nitrosated at other sites in addition to thiols and metals. In addition, Hb can be nitrated. Hbs which have been nitrosated and/or nitrated at multiple different types of

nucleophilic sites (termed polynitrosated, that is, having NO equivalents added to other nucleophilic sites as well as to thiols; or polynitrated, respectively) will permit transnitrosation reactions and the release of NO and its biological equivalents in the circulatory system at different rates and engendering different potencies.

These and other nitrosation and nitration reactions can cause oxidation of the heme Fe to some extent. However, some minor degree of oxidation is acceptable. The nitrosated Hb is still be useful as a therapeutic agent if oxidized to a minor extent. For applications where the the NO-delivering function, rather than the O_2-delivering function of nitrosated Hb, is more desirable, extensive oxidation of the heme Fe is acceptable.

If it is desired to avoid oxidation of the heme Fe, it is possible to remove the heme, perform the necessary chemical reactions upon the protein to nitrosate to the extent desired, and replace the heme into the modified hemoglobin product. (See, for removing and replacing the heme, Antonini, E. and Brunori, M., *Hemoglobin and Myoglobin in their Reactions with Ligands*, Elsevier, New York, 1971.)

In addition to the nitrosating under conditions that do not oxidize the heme, such as brief exposure to low molecular weight RSNOs, as illustrated in Examples 1 and 2, alternative methods can be used to produce nitrosated hemoglobin in which the heme Fe is not oxidized. For instance, it is possible to produce by recombinant methods α and β globin chains, nitrosate them to the extent desired, then assemble the chains with heme to form a functional, nitrosated tetramer. (See, for example, European Patent Application EPO 700997, published March 13, 1996, filed May 10, 1990, "Production in bacteria and yeast of hemoglobin and analogues thereof.")

Another alternative method to nitrosate the α and β globin chains without producing a form of metHb as the end product, is to nitrosate the intact Hb molecule to the extent desired, thereby allowing the heme Fe to be oxidized, then reduce the heme Fe by treating the nitrosated Hb with either methemoglobin reductase or a cyanoborohydride such as sodium cyanoborhydride.

The term hemoglobin or Hb as used herein includes variant forms such as mutant forms, chemically modified forms, genetically altered forms, such as fusion proteins, and truncated forms. It also includes Hbs of all animal species and variant forms thereof. The biological and/or chemical properties of these variant Hbs may be different from those of hemoglobins which are found naturally occurring in animals.

It will be appreciated that NO exists in biological systems not only as nitric oxide gas, but also in various redox forms and as biologically active adducts of nitric oxide such as S-nitrosothiols, which can include S-nitrosoproteins, S-nitroso-amino acids and other S-nitrosothiols (Stamler, J.S. *Cell 78*:931-936 (1994)).

A blood substitute can be a biologically compatible liquid which performs one or more functions of naturally occurring blood found in a mammal such as oxygen carrying and/or delivery, NO carrying and/or delivery, and the scavenging of free radicals. A blood substitute can also comprise one or more components of such a liquid which, when infused into a mammal, perform one or more functions of naturally occurring blood. Examples of blood substitutes include preparations of various forms of hemoglobin. Such preparations may also include other biologically active components, such as a low molecular weight thiol, nitrosothiol or NO donating agents, to allow transnitrosation.

The compounds and therapeutic preparations of this invention to be used in medical treatment are intended to be used in therapeutically effective amounts, in suitable compositions, which can be determined by one of skill in the art. Modes of administration are those known in the art which are most suitable to the affected site or system of the medical disorder. Suitable compositions may include carriers, stabilizers or inert ingredients known to those of skill in the art, along with biologically active component(s).

The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of nitrosated Hb and/or nitrosating agent which is effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges for effective amounts of each compound to be administered is within the skill of one in the art. Research animals such as dogs, baboons or rats can be used to determine dosages. Generally, dosages required to provide effective amounts of the composition or preparation, and which can be adjusted by one of ordinary skill in the art, will vary, depending on the age, health, physical condition, sex, weight, extent of disease of the recipient, frequency of treatment and the nature and scope of the desired effect. Dosages for a particular patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol). Suitable pharmaceutical carriers can be combined with with active ingredients employed in a therapeutic composition, if necessary.

The present invention is further and more specifically illustrated in the following examples.

EXAMPLES

Example 1: Interactions of NO and RSNO with Hb

It was observed that naturally occurring N-oxides, such as NO and RSNOs (Gaston, B., *et al.*, (1993); Scharfstein, J.S., *et al.*, *J. Clin. Invest.*, *94*:1432-1439 (1994); Clancy, R.M., *et al.*, *Proc. Natl. Acad. Sci USA*, *91*:3680-3684 (1994)), differed markedly in their reactions with Hb. NO bound very rapidly to deoxyHb (Hb[FeII]), forming relatively stable Hb[FeII]NO complexes (Figure 1A), and converted oxyHb (Hb[FeII]O_2) to methemoglobin (Hb[FeIII]) and nitrate (Figure 1B), confirming previous reports (Olson, (1981); Toothill, C., *Brit. J. Anaesthy.* *39*:405-412 (1967)). In contrast, RSNOs were found to participate in transnitrosation reactions with sulfhydryl groups of Hb, forming S-nitrosohemoglobin (SNO-Hb), and did not react with the heme centers of either deoxyHb or Hb(FeII)O_2 (Figures 1C and 1D).

A. Interaction of NO with deoxyHb

Conversion of deoxyHb (Hb[FeII]) to Hb(FeII)NO is observed upon incubation of Hb(FeII) with increasing concentrations of nitric oxide. *a.* Deoxy Hb. *b, c, d.* Reaction mixtures of NO and Hb(FeII) in ratios of 1:1, 2:1 and 10:1, respectively. The reaction product Hb(FeII)NO formed essentially instantaneously on addition of NO (*i.e.* within instrument dead time).

B. Interaction of NO with oxyHb

Conversion of oxyHb (Hb[Fe[II]O_2) to metHb (HbFe[III]) is observed upon incubation of oxyHb with increasing concentrations of NO. *a.* oxy Hb. *b, c, d.* Reaction mixtures containing NO and oxyHb in ratios of 1:1, 2:1 and

10:1, respectively. Methemoglobin formation occurred instantaneously on addition of NO (*i.e.* within instrument dead time).

C. Interaction of S-nitrosothiols with deoxyHb

Conversion of Hb(FeII) to SNO-Hb(FeII) is observed upon incubation of either GSNO (shown) or S-nitrosocysteine (CYSNO) with deoxy Hb. There is little (if any) interaction of RSNO with the heme functionalities of Hb. *a.* deoxyHb. *b, c, d.* Reaction mixtures of GSNO and Hb(FeII) in ratios of 1:1, 2:1 and 10:1, respectively. Spectra were taken after 60 min of incubation in *b, c,* and 15 min in *d.* Further analysis of reaction products revealed the formation of moderate amounts of SNO-Hb in all cases. Yields of SNO-Hb (S-NO/Hb) in *b, c,* and *d* at 60 min were 2.5%, 5% and 18.5%, respectively. (See Figure 1D and Figure 2A.)

D. Interaction of S-nitrosothiols with oxyHb

Conversion of Hb(FeII)O_2 to SNO-Hb(FeII)O_2 is observed upon incubation of either GSNO (shown) or CYSNO with oxyHb. There is little (if any) reaction of GSNO (or CYSNO) at the heme centers of Hb(FeII)O_2. Specifically, the capacity for O_2 binding to heme is unaffected by RSNOs. *a.* oxyHb. *b, c, d.* Reaction mixtures of GSNO and oxyHb in ratios of 1:1, 2:1, and 10:1, respectively. Spectra were taken after 60 min of incubation in the spectrophotometer. Further analysis of reaction products revealed the formation of SNO-Hb in all cases. Yields of SNO-Hb in spectra *b, c* and *d* were 5%, 10% and 50% (S-NO/Hb), respectively. In 5 other determinations, the yield of S-NO/Hb was 0.37 ± 0.06 using GSNO (pH 7.4, 10-fold excess over Hb) and ~2 SNO/tetramer (1.97 ± 0.06) using CYSNO (*vida infra*). These last data are in agreement with reports that human HbA contains 2 titratable SH groups.

Methods

Human HbA_0 was purified from red cells as previously described (Kilbourn, R.G., *et al.*, *Biochem. Biophy. Res. Comm.*, *199*:155-162 (1994)). Nitric oxide solutions were rigorously degassed and purified according to standard procedure (Beckman, J. S., *et al.*, *Methods in Nitric Oxide Research*, Feelisch and Stamler, eds., Wiley Chichester, U.K. (1996)) and saturated solutions were transferred in air tight syringes. Deoxygenation of Hb was achieved by addition of excess dithionite (NO studies) or by reduction of $Hb(FeII)O_2$ through evacuation in Thunberg tubes (RSNO studies; as RSNOs react with dithionite). RSNOs were synthesized as previously described (Gaston, B., *et al.*, (1993); Arnelle and Stamler, (1995)) Incubations with HbA_0 were made in phosphate buffer, pH 7.4, 0.5 mM EDTA. Quantifications of SNO-Hb were made according to the method of Saville (Gaston, B., *et al.*, (1993); Stamler, J.S., *et al.*, *Proc. Natl Acad. Sci. USA*, *90*:444-448 (1992)) after purification of protein with Sephadex G-25 columns. The Saville method, which assays free NO_x in solution, involves a diazotization reaction with sulfanilamide and subsequent coupling with the chromophore N-(naphthyl)ethylenediamine. No low molecular weight S-NO complexes survived this purification and all activity was protein precipitable. The reactions and spectra were carried out using a Perkin Elmer UV/Vis Spectrometer, Lambda 2S.

Example 2: Allosteric Function of O_2 in Regulation of Hb S-nitrosylation

Oxygenation of Hb is associated with conformational changes that increase the reactivity of cysβ93 to alkylating reagents (Garel, C., *et al.*, *J. Biochem.*, *123*:513-519 (1982); Jocelyn, P.C., *Biochemistry of the SH Group*, Academic Press, London, p.243 (1972); Craescu, C.T., *et al.*, *J. Biol. Chem.*, *261*:14710-14716 (1986)). The

physiological importance of this effect was never established. It was observed here that rates of S-nitrosylation of Hb were markedly dependent on conformational state. In the the oxy conformation (R state), S-nitrosylation was more rapid than in the deoxy conformation (T state) (Figure 2A). The rate of S-nitrosylation was accelerated in both conformations by alkaline conditions (*i.e.* rate at pH 9.2 > pH 7.4), which would tend to expose the cysβ93 that is otherwise screened from reaction by the C-terminal histidine 146β. The salt bridge (asp β94 --- his β146) tying down the histidine residue is loosened at high pH. These data suggest that the increase in thiol reactivity associated with the R state derives, at least in part, from improved NO access rather than a conformation-induced change in pK.

A. Oxygenation accelerates S-nitrosylation of Hb.

Rates of Hb S-nitrosylation by S-nitrosocysteine (CYSNO) are faster in the oxy conformation (Hb[FeII]O_2) than in the deoxy state (Hb[FeII]).

Methods

Incubations were performed using 10-fold excess CYSNO over protein (50 μM) in aerated 2% borate, 0.5 mM EDTA (oxyHb), or in a tonometer after rapid O_2 evacuation (deoxyHb). At shown times, samples were rapidly desalted accross G-25 columns (preequilibrated with phosphate buffered saline, 0.5 mM EDTA, pH 7.4) to remove CYSNO, and analyzed for SNO-Hb by the method of Saville (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA, 89*:444-448 (1992)).

B. Deoxygenation accelerates denitrosylation of Hb

Rates of RSNO decomposition (and transfer) are much faster in the deoxy conformation [SNO-Hb(FeII)] than in the

oxy state [SNO-Hb(FeII)O_2]. The decomposition of SNO-Hb(FeII) is further accelerated by the presence of excess glutathione. Within the dead time of our measurements (~15 sec) a major fraction of SNO-Hb(FeII) was converted to GSNO.

Methods

Hbs in PBS (0.5 mM EDTA, pH 7.4) were incubated in air (oxy) or in a tonometer previously evacuated of O_2 (deoxy). SNO-Hb(FeII)O_2 decomposition was determined by the method of Saville (Saville, B., *Analyst 83*:670-672 (1958)). Spontaneous decomposition of SNO-Hb(FeII) was followed spectrophotometrically by formation of Hb(FeII)NO. Transnitrosation reactions with glutathione were performed by addition of 100-fold excess glutathione over protein (50 μM), immediate processing of the reaction mixture under anaerobic conditions followed by rapid TCA precipitation, and analysis of RSNO in the supernatant. Rates of NO group transfer were too rapid to measure accurately by the standard methods used in this study.

Example 3: NO-related Interactions with Cysteine Residues of Hb in Physiological Systems

Given that Hb is largely contained within red blood cells, potential mechanisms by which S-nitrosylation of intracellular protein might occur were explored. Incubation of oxygenated rat red blood cells with S-nitrosocysteine resulted in very rapid formation of intracellular SNO-Hb(FeII)O_2 (Figure 3A). Rapid oxidation of Hb was not observed under these conditions. Intraerythrocytic SNO-Hb also formed when red blood cells were treated with S-nitrosohomocysteine or S-nitrosocysteinylglycine, but not with S-nitrosoglutathione (GSNO). Thus, erythrocyte access of RSNOs is thiol group

specific. Exposure of oxygenated red blood cells to NO resulted primarily in metHb formation.

Endothelium-derived relaxing factor (EDRF) and hemoglobin

Hb-mediated inhibition of endothelium-dependent relaxations is commonly used as a marker of NO responses. Inasmuch as reactions with either metal or thiol centers of Hb should lead to attenuated NO/EDRF (endothelium-derived relaxing factor) responses, we attempted to elucidate the molecular basis of inhibition. Hb preparations in which β93 thiol groups had been blocked with N-ethylmaleimide (NEM) or the hemes blocked by cyanmet (FeIIICN)-derivitization were studied in an aortic ring bioassay, and their activities compared with that of native Hb. Both cyanmet-Hb and NEM-Hb caused increases in vessel tone and attenuated acetylcholine (EDRF)-mediated relaxations (Figure 3B). However, native Hb was significantly more effective than either of the modified Hb preparations (Figure 3B). Taken in aggregate, these studies suggest that both the thiol and metal groups of Hb contribute to its NO-related activity. To verify formation of an S-nitrosothiol in Hb, a bioassay was used in which 2 cm segments of thoracic aorta were interposed in Tygon tubing, through which 3 cc of Krebs solution containing Hb (4μM) and ACh (2μM) were circulated by roller pump (1.5 cc/min x 5 min). Analysis of the effluent (Gaston, B., *et al.*, (1993)) revealed the formation of SNO-Hb (20 ± 4 nM) in 5 of 5 experiments.

A. S-nitrosylation of intraerythrocytic Hb

Incubation of rat erythrocytes with S-nitrosocysteine (equimolar to heme (5mM); phosphate buffer pH 7.4, 25°C) leads to rapid formation of intracellular SNO-Hb(FeII)O_2. MetHb does not form rapidly. Separation of intracellular RSNOs across G-25 columns reveals that only a small

percentage exists as low molecular weight S-nitrosothiol (e.g. GSNO) at most time points. By 60 min, 3 of the 4 available SH groups of Hb are S-nitrosylated (note that rat Hb contains 4 reactive SH groups). Inset shows spectra of SNO-Hb isolated from rat erythrocytes and related analyses. Spectrum *A* is that of SNO-Hb isolated from erythrocytes following G-25 chromatography. Treatment of *A* with dithionite results in reduction of the S-NO moiety, liberating free NO which is autocaptured by deoxy Hb, forming Hb(FeII)NO (note that dithionite simultaneously deoxygenates Hb) (spectrum *C*). This spectrum (*C*) reveals a stoichiometry of ~3 S-NOs per tetramer. The spectrum of Hb(FeII)NO containing 4 NO's per tetramer is shown for comparison (inset, spectrum *B*).

Methods

At shown intervals, red blood cells were pelleted rapidly by centrifugation, washed three times, lysed in deionized water at 4°C, and the cytosolic fraction subjected to rapid desalting across G-25 columns. Intracellular SNO-Hb was measured by the method of Saville (Gaston, B., *et al.*, (1992); Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA, 89*:444-448 (1992)), and confirmed spectroscopically (inset) as described above.

B. Molecular Basis of EDRF/Hb Interaction.

The effects of native Hb on EDRF responses were compared with Hb preparations in which the thiol or heme centers had been blocked by alkylation or cyanmet derivitization, respectively. All preparations of Hb elicited contractions; however, those of native Hb (in which both SH and metal centers are free for interaction) were most pronounced. Likewise, acetylcholine (ACh) mediated relaxations were most effectively inhibited by native Hb. Relaxations were inhibited to lesser degrees by

cyanmet Hb (CN-Hb)(in which hemes were blocked from reaction) and NEM-Hb (in which thiol groups were alkylated by N-ethylmaleimide). These data illustrate that both heme and β93SH groups of Hb contribute to reversal of EDRF responses. Direct measurement of SNO-Hb, formed from EDRF under similar conditions, is described in the text.

Methods

Descending rabbit thoracic aorta were cut into 3 mm rings and mounted on stirrups attached to force transducers (model FT03, Grass Instruments, Quincy, MA) for measurement of isometric tone. The details of this bioassay system have been previously described (Stamler, J.S., *et al.*, *Proc. Natl. Acad. Sci. USA, 89:*444-448 (1992)). Cyanmet Hb was prepared from human HbA according to published protocols (Kilbourn, R.G. et al. *Biochem. Biophy. Res. Comm., 199:*155-162, (1994)). Alkylation of HbA with N-ethylmaleimide was followed by desalting across G-25 Sephadex to remove excess NEM. Removal of unmodified Hbcysβ93 was achieved by passage through Hg-containing affinity columns. The alkylation of free SH groups was verified using 5,5'-dithio-bis[2-nitrobenzoic acid].

Table 1

ADDITIONS	% INCREASE IN TENSION (↓)	% ACh RELAXATION (↑)
Hb (1 μM)	40.8 ± 2.3 (n=7)	31.9 ± 6.9 (n=7)
NEM-Hb (1 μM)	29.4 ± 1.3 **(n=7)	60.5 ± 3.9 * (n=7)
CN-Hb (1 μM)	12.9 ± 3.0 ** (n=6)	80.7 ± 1.0 ** † (n=4)
ACh (1 μM)		98.3 ± 0.6 (n=10)

*, P<0.01; **, P<0.001, Compared to Hb; †, P<0.001, Compared to ACh

Example 4: Transduction of SNO-Hb Vasoactivity

Arterial red blood cells contain two physiologically important forms of hemoglobin: Hb(FeII)O_2 and Hb(FeIII) (Antonini *et al.* (1971)). Arterial-venous differences in the S-nitrosothiol content of intraerythrocytic Hb suggest that the NO group is released during red cell transit. Such findings raise the possibility of functional consequences, perhaps influenced by the redox state of heme and its occupation by ligand. Intriguingly, SNO-Hb(FeII)O_2 was found to possess modest NO-like activity when tested in a vascular ring bioassay. Specifically, the contraction elicited by SNO-Hb(FeII)O_2 was less than that of native Hb(FeII)O_2, indicating that S-nitrosylation partially reverses the contractile effects of Hb (Figure 4A). By comparison, SNO-Hb(FeIII) was found to be a vasodilator (Figure 4A). Notably, free NO was devoid of relaxant activity in the presence of Hb(FeII)O_2 or Hb(FeIII) (not shown).

Red blood cells contain millimolar concentrations of glutathione. As equilibria among RSNOs are rapidly established through RSNO/thiol exchange (Arnelle, D.R. and Stamler, J.S., *Arch. Biochem. Biophy., 318:*279-285 (1995)), the vasoactivity of SNO-Hb was reassessed in the presence of glutathione. Figure 4B illustrates that glutathione potentiated the vasodilator activity of both SNO-Hb(FeII)O_2 and SNO-Hb(FeIII). GSNO formation under these conditions (confirmed chemically and in bioassay experiments) appeared to fully account for this effect. Further kinetic analyses revealed that transnitrosation involving glutathione was more strongly favored in the equilibrium with SNO-Hb(FeIII) than SNO-Hb(FeII)O_2 (Figure 4C). Given the findings of steady-state levels of SNO-Hb in red blood cells (Table 2 and Figure 3A), these results suggest that 1) the equilibrium between naturally occurring RSNOs and

Hb(cysβ93) lies toward SNO-Hb under physiological conditions; 2) that transnitrosation reactions involving SNO-Hb and GSH are likely to occur within red blood cells (in these studies, low molecular weight RSNOs have been found in erythrocytes loaded with SNO-Hb); and 3) that oxidation of the metal center of Hb shift the equilibrium toward GSNO, thereby potentially influencing bioactivity.

Additional mechanisms of NO group release from SNO-Hb were sought. Arterial-venous differences in levels of SNO-Hb raised the possibility that S-NO bond stability may be regulated by the changes in Hb conformation accompanying deoxygenation. To test this possibility, the rates of NO group release from SNO-Hb(FeII)O_2 and SNO-Hb(FeIII) were compared. Deoxygenation was found to enhance the rate of SNO-Hb decomposition (Figure 2B). These rates were accelerated greatly by glutathione in a reaction yielding GSNO (Figure 2B). The results illustrate that O_2-metal interactions influence S-NO affinity, and suggest a new allosteric function for Hb.

For SNO-Hb to be of physiological importance it must transduce its NO-related activity across the erythrocyte membrane. This possibility was explored by incubating erythrocytes containing SNO-Hb in physiologic buffer, and measuring the accumulation of extracellular RSNOs over time. Figure 4D illustrates that red blood cells export low molecular weight (trichloroacetic acid precipitable) S-nitrosothiols under these conditions. Importantly, the degree of hemolysis in these experiments was trivial (<0.5%), and correction for lysis did not significantly impact on rates of RSNO release. These results establish that an equilibrium exists between low molecular weight and protein RSNOs within the red cell, and that intracellular location is unlikely to be a limiting factor in the transduction of such NO-related activity to the vessel wall.

A. Concentration-effect responses of different SNO-Hb preparations.

Contractile effects of Hb(FeII)O_2(▲) are shown to be partially reversed by S-nitrosylation (SNO-Hb[FeII]O_2(■); P = 0.02 by ANOVA vs Hb(FeII)O_2) (See Figure 4A.). Oxidation of the metal center of SNO-Hb (SNO-Hb[FeIII](●)) converts the protein into a vasodilator (P < 0.0001 by ANOVA vs. SNO-Hb[FeII]O_2), with potency comparable to that of other S-nitrosoproteins (Stamler, J.S., *et al., Proc. Natl. Acad. Sci. USA, 89:*444-448 (1992)). The contractile properties of Hb(FeIII) are shown for comparison (□); n = 6-17 for each data point.

Methods

Details of the vessel ring bioassay have been published (Stamler, J.S., *et al., Proc. Natl. Acad. Sci. USA 89:*444-448 (1992)). SNO-Hb(FeII)O_2 preparations were synthesized with 10-fold excess S-nitrosocysteine (CYSNO) over Hb(FeII)O_2 protein (2% borate, 0.5 mM EDTA, ~15 min incubation), after which desalting was performed across Sephadex G-25 columns. CYSNO was synthesized in 0.5 N HCl, 0.5 mM EDTA and then neutralized (1:1) in 1 M phosphate buffer containing 0.5 mM EDTA. SNO-Hb(FeIII) preparations followed a similar protocol, but used Hb(FeIII) as starting material. The latter was synthesized by treatment of Hb(FeII)O_2 with excess ferricyanide, followed by desalting accross G-25 columns. SNO-Hb concentrations were verified spectroscopically and the S-nitrosothiol content was determined by the method of Saville (Stamler, J.S., *et al., Proc. Nat. Acad. Sci USA 89:*444-448 (1992)). The S-NO/tetramer stoichiometry for both SNO-Hb preparations was ~2. Oxidation of the heme was undetectable by uv-spectophotometric methods.

B. Potentiation of SNO-Hb effects by Glutathione

Addition of glutathione (100 μM) to bioassay chambers potentiates the dose-response to both SNO-Hb(FeII)O_2(■) and SNO-Hb(FeIII)(●) (See Figure 4B. n = 6-12; p < 0.0001 for both by ANOVA, compared with the respective tracings in a). Glutathione had a transient affect on baseline tone in some experiments, and did not significantly influence the response to Hb(FeII)O_2 (▲).

C. Transnitrosation between SNO-Hb and Glutathione

Rates of NO group transfer from SNO-Hb (100μM) to glutathione (10 mM) are displayed for SNO-Hb(FeII)O_2 (oxy) and SNO-Hb(FeIII) (met) (n=5). Data are presented as the amount of GSNO formed relative to the starting SNO-Hb concentration. The transfer is more rapid for SNO-Hb(FeIII) than SNO-Hb(FeII)O_2 ($p<0.002$ by ANOVA), suggesting that the GSNO/SNO-Hb equilibrium is shifted toward GSNO by formation of met Hb.

Methods

Thiol/SNO-Hb exchange, forming GSNO, was verified chemically (Stamler, J.S., *et al., Proc. Natl Acad. Sci. USA, 89*:444-448 (1992)) following trichloroacetic acid precipitation (n=5). These results were verified in separate experiments by measuring the residual SNO-Hb concentration, following separation of reaction mixtures accross G-25 columns.

D. Export of S-nitrosothiols by red blood cells

Human red blood cells containing SNO-Hb are shown to export low molecular weight RSNOs over time. Hemolysis, which ranged from 0-<0.5% over one hour and did not correlate with rates of RSNO release, could account for only a trivial fraction of the measured extracellular RSNO.

Methods

Packed human red blood cells were obtained by centrifugation, washed, and resuspended in phosphate buffered saline containing 5 mM SNOCYS (0.5 mM EDTA, pH 7.4) for one hour. This results in a red cell preparation containing SNO-Hb ($FeIIO_2$/FeIII mixture) with a stoichiometry of 0.5 S-NO/tetramer. The red blood cells were then washed repeatedly to remove residual CYSNO (verified), and incubated in Krebs' solution (1:4). The accumulation of extracellular RSNO was measured over time by the method of Saville (Saville, B., *Analyst*, *83*:670-672 (1958)). Hemolysis was determined by spectral analysis of red blood cell supernatants following centrifugation.

Example 5: SNO-Hb Bioactivity *In Vivo*

Systemic administration of cell-free Hb results in hypertensive responses which have been attributed to NO scavenging by the heme (Vogel, W.M., *et al.*, *Am. J. Physiol. 251*:H413-H420 (1986); Olsen, S.B., *et al.*, *Circulation 93*:329-332 (1996)). To determine if SNO-Hb is free of this adverse affect, and to explore if *in vitro* mechanisms of NO release extend to the *in vivo* circumstance, we compared responses to Hb and SNO-Hb infused as a bolus into the femoral vein of anesthetized rats. As illustrated in Figure 5, Hb(FeII)O_2 (200 nmol/kg) caused an increase in mean arterial pressure of 20 ± 3 mm Hg ($n = 4$; $P < 0.05$). In contrast, SNO-Hb(FeII)O_2 did not exhibit hypertensive effects and SNO-Hb(FeIII) elicited hypotensive responses (Figure 5). Thus, the profiles of these compounds *in vivo* closely resemble those seen *in vitro* (Figure 4A). Moreover, to demonstrate that the physiological responses of red cells are comparable to those of cell-free Hb preparations, erythrocytes containing SNO-Hb were injected into the femoral vein of rats pretreated with L-NMMA (50 mg/kg) to deplete endogenous

RSNOs. At levels of SNO-Hb comparable to those found in the normal rat (0.1-0.5 μM), SNO-Hb containing red blood cells elicited hypotensive responses (8 ± 1 mm Hg; mean ± SEM; n=9), whereas native (SNO-Hb depleted) red blood cells did not (P=0.001). These changes in mean blood pressure of ~ 10% are on the order of those that differentiate normotension from hypertension in man, and in the therapeutic range of some antihypertensive regimens. The effects of both Hb and SNO-Hb -- whether cell-free or contained within red cells -- were transient, suggesting that S-nitrosylation of Hb and metabolism of SNO-Hb may be occurring *in vivo*, with consequent restoration of blood pressure. The bioactivity of SNO-Hb in blood, where S-NO/heme stoichiometries approach 1:50,000, is a dramatic illustration of the resistence of this NO-related activity to Hb(Fe) inactivation.

In vivo effects of cell-free Hb and SNO-Hbs

Administration of 2-200 nmol/kg $Hb(FeII)O_2$ (as a bolus) into the femoral vein of a Sprague-Dawley rat is shown to increase mean arterial pressure in a dose-dependent manner. At 200 nmol/kg, mean arterial pressure increased by 25 mm Hg (20 ± 3 mm Hg; n = 4; $P < 0.05$). Elevations in blood pressure reversed within 10-15 min. SNO-$Hb(FeII)O_2$ infusions (over the same dose range) are shown to ameliorate $Hb(FeII)O_2$-induced hypertension without causing overt changes in blood pressure. A similar response was seen at higher doses. By comparison, SNO-Hb(FeIII) infusions caused a significant fall in mean arterial pressure (pre 108 ± 4 mm Hg; post 74 ± 6 mm Hg, n = 5; $P < 0.05$) at the highest dose (200 nmol/kg). Hypotensive responses tended to be transient with blood pressure normalizing over 10 minutes. A fall in blood pressure was also seen with injection of erythrocytes containing SNO-Hb.

Methods

Rats were anesthetized by intraperitoneal injection of pentobarbital and the femoral arteries and veins accessed by local cut down. The artery was then cannulated and the blood pressure monitored continuously using a Viggo Spectramed pressure transducer attached to a Gould recorder. An IBM PC (DATA Q Codas) was used for data acquisition.

Example 6: Loading of Red Blood Cells With S-Nitrosothiols

Incubation of rat erythrocytes with S-nitrosocysteine (equimolar to heme (5mM); phosphate buffer pH 7.4, 25°C) leads to rapid formation of intracellular S-nitrosothiols. MetHb does not form rapidly. Separation of cell content across G-25 columns establishes the formation of intraerythrocytic low molecular weight S-nitrosothiol, e.g. S-nitrosoglutathione, (GSNO). By 2 min., one can achieve as much as millimolar GSNO.

Method for assay of RSNO

S-nitrosocysteine (5 mM) treated red blood cells are pelleted rapidly by centrifugation, washed three times, lysed in deionized water at 4°C, and the cytosolic fraction subjected to rapid desalting across G-25 columns. Intracellular RSNO is measured by the method of Saville and can be confirmed spectroscopically.

Effects on blood pressure from loaded red blood cells

Red blood cells treated with S-nitroscysteine (to produce SNO-RBCs) and introduced into the femoral vein of a Sprague-Dawley rat decreased mean arterial pressure in a dose-dependent manner. For red blood cells in which SNO-Hb was assayed at 0.3 μM (the endogenous in vivo SNO-Hb concentration), arterial pressure decreased by 8 ± 1 mm Hg

(mean ± SEM for 9 experiments; $p < 0.001$ compared to untreated red blood cell controls). For red blood cells in which SNO-Hb was assayed at 0.5 μM, arterial pressure decreased by 10 mm Hg. For red blood cells in which SNO-Hb was assayed at 0.1 μM (a sub-endogenous SNO-Hb concentration), arterial pressure decreased by 6 mm Hg. The administration of untreated red blood cells caused no effect or a slight increase in arterial blood pressure. Administration of L-monomethyl-L-arginine (L-NMMA; 50 mg/kg) caused an increase in blood pressure of about 20 mm Hg. Changes in blood pressure from a bolus administration of loaded red blood cells lasted 15-20 minutes.

Further methods

Rats were anesthetized by intraperitoneal injection of pentobarbital and the femoral arteries and veins accessed by local cut down. The artery was then cannulated and the blood pressure monitored continuously using a Viggo Spectramed pressure transducer attached to a Gould recorder. An IBM PC (DATA Q Codas) was used for data acquisition.

Example 7: Effects of SNO-Hb on Coronary Vasodilation, Coronary Flow and Blood Pressure

SNO-Hb was synthesized as described in Example 4A. Completion of the reaction was determined as described in Example 4A. Twenty-four healthy mongrel dogs (25-30 kg) were anesthetized with intravenous thiamylal sodium (60-80 mg/kg) and subjected to left thoracotomy in the fourth intercostal space. The left circumflex coronary artery distal to the left atrial appendage was minimally dissected. A pair of 7-MHz piezoelectric crystals (1.5 X 2.5 mm, 15-20 mg) was attached to a Dacron backing and sutured to the adventitia on opposite surfaces of the dissected vessel segment with 6-0 prolene. Oscilloscope

monitoring and on-line sonomicrometry (sonomicrometer 120-2, Triton Technology, San Diego, CA) were used to ensure proper crystal position. A pulse Doppler flow probe (10 MHz, cuff type) was implanted distal to the crystals. An inflatable balloon occluder was also placed distal to the flow probe. All branches of the circumflex artery between the crystals and the occluder were ligated. Heparin sodium-filled polyvinyl catheters were inserted into the left ventricular cavity via the apex, into the left atrium via the atrial appendage, and into the ascending aorta via the left internal thoracic artery. The catheters, tubing, and wires were tunnelled to a subcutaneous pouch at the base of the neck.

After a 10 to 15 day recovery period, the catheters and wires were exteriorized under general anesthesia, and 2-3 days later, each dog was given a bolus injection of SNO-Hb (0.4 mg) to evaluate vascular response. Two dogs that demonstrated <5% dilation of epicardial coronary vessels were excluded from subsequent studies, and two were excluded because of other technical reasons.

Dogs were trained and studied while loosely restrained and lying awake in the lateral recumbent position. The laboratory was kept dimly illuminated and quiet. Aortic pressure, left ventricular end-diastolic pressure dP/dt external coronary diameter and coronary flow were monitored continuously. In 10 dogs, 0.1 ml of SNO-Hb solution, 50 nM/kg, was injected via the left atrial catheter. To verify potential effects of solvent on vasculature, 0.1 ml injections of 30% ethanol in distilled water were given as vehicle control. Between injections, phasic coronary blood flow and coronary artery diameter were allowed to return to preinjection levels (minimum 15 minutes). Allowing a 15 minute period between injections resulted in no modification of repeated does injections. To assess the direct and potential flow mediated indirect vasodilation

effects of SNO-Hb on the conductance vessels, the dose was repeated in 6 of 10 dogs with partial inflation of the adjustable occluder to maintain coronary blood flow at or slightly below preinjection levels. The response to acetylcholine chloride (Sigma Chemical) was assessed in another group of 10 dogs following a similar protocol to that used for SNO-Hb.

Epicardial coronary diameter, coronary blood flow, heart rate, and aortic and left ventricular end-diagnostic pressures were compared before and after each SNO-Hb injection. The maximum changes in coronary dimension and blood flow were expressed as a function of increasing doses of SNO-Hb. The response of coronary dimension to increasing doses followed a characteristic sigmoid dose-response curve that could be described by the following equation

$$Effect = \frac{maximal\ effect\ x\ dose}{K_D + dose}$$

where K_D is the drug-receptor complex dissociation constant and is the dose at which 50% of the maximum response (EC_{50}) is achieved. In each animal, a nonlinear least-squares regression ($r^2 > 0.90$) was performed on the dose-response data. The regression was constrained to the above equation. From the regression, values for maximum response and K_D were obtained for each individual animal. The mean of these values was then calculated to obtain an average K_D and maximum response for the study group. These values were used to generate a mean curve, which was plotted with the mean dose-response values. (See Figures 6A-6F.)

Example 8: Endogenous Levels of S-nitrosohemoglobin and Nitrosyl(FeII)-Hemoglobin in Blood

To determine if SNO-Hb is naturally occuring in the blood, and if so, its relationship to the O_2 transport capacity and nitrosylated-heme content of red cells, an analytical approach was developed to assay the S-nitrosothiol and nitrosyl-heme content of erythrocytes (Table 2). Arterial blood was obtained from the left ventricle of anesthetized rats by direct puncture and venous blood was obtained from the jugular vein and inferior vena cava. Hb was then purified from red cells and assayed for RSNO and (FeII)NO content. Arterial blood contained significant levels of SNO-Hb, whereas levels were virtually undetectable in venous blood (Table 2). Measurements made 45 minutes after infusion of the NO synthase inhibitor N$^{\omega}$-monomethyl-L-arginine (L-NMMA) (50 mg/kg), showed a depletion of SNO-Hb as well as total Hb-NO (82 and 50 ± 18%, respectively; n = 3-5; $p < 0.05$). These data establish the endogenous origin of SNO-Hb, although some environmental contribution is not excluded. The arterial-venous distribution seen for SNO-Hb was reversed in the case of Hb(FeII)NO, which was detected in higher concentrations in partially deoxygenated (venous) erythrocytes (Table 2). Accordingly, the proportion of nitrosylated protein thiol and heme appears to depend on the oxygenation state of the blood. Consistent with these findings, Wennmalm and coworkers have shown that Hb(FeII)NO forms mainly in venous (partially deoxygenated) blood (Wennmalm, A., *et al.*, *Br. J. Pharmacol. 106(3)*:507-508 (1992)). However, levels of Hb(FeII)NO *in vivo* are typically too low to be detected (by EPR) and SNO-Hb is EPR-silent (*i.e.*, it is not paramagnetic). Thus, photolysis-chemiluminesence represents an important technological advance, as it is the first methodology

capable of making quantitative and functional assessments of NO binding to Hb under normal physiological conditions.

Methods

Blood was obtained from the left ventricle (arterial) and jugular vein (venous) of anesthetized Sprague-Dawley rats. Comparable venous values were obtained in blood from the inferior vena cava. Red blood cells were isolated by centrifugation at 800 g, washed three times in phosphate buffered saline at 4°C, lysed by the addition of 4-fold excess volume of deionized water containing 0.5 mM EDTA, and desalted rapidly across G-25 columns according to the method of Penefsky at 4°C. In 24 rats, Hb samples were divided in two aliquots which were then treated or not treated with 10-fold excess $HgCl_2$ over protein concentration as measured by the method of Bradford. Determinations of SNO-Hb and Hb(FeII)NO were made by photolysis-chemiluminescence as described below. In 12 additional rats, further verification of the presence of SNO-Hb was made by assaying for nitrite after $HgCl_2$ treatment. Specifically, samples (with and without $HgCl_2$) were separated across Amicon-3 (Centricon filters, m.w. cut off 3,000) at 4°C for 1 h, and the low molecular weight fractions collected in airtight syringes containing 1 μM glutathione in 0.5 N HCl. Under these conditions, any nitrite present was converted to S-nitrosoglutathione, which was then measured by photolysis-chemiluminescence (detection limit ~ 1 nM). SNO-Hb was present in all arterial samples, and levels determined by this method (286 ± 33 nM) were virtually identical to and not statistically different from those shown in Table 2. In venous blood, SNO-Hb was undetectable (0.00 ± 25 nM); levels were not statistically different from those given above.

Method for assay of S-nitrosohemoglobin

A highly sensitive photolysis-chemiluminescence methodology was employed. A somewhat similar assay has been used for measuring RSNOs (S-nitrosothiols) in biological systems (Gaston, B., *et al.*, (1993); Stamler, J.S., *et al.*, (1992)). The method involves photolytic liberation of NO from the thiol, which is then detected in a chemiluminesence spectrometer by reaction with ozone. The same principle of operation can be used to cleave (and measure) NO from nitrosyl-metal compounds (Antonini, E. and Brunori, M. In *Hemoglobin and Myoglobin in Their Reactions with Ligands*, American Elsevier Publishing Co., Inc., New York, pp. 29-31 (1971)). With adjustment of flow rates in the photolysis cell, complete photolysis of the NO ligand of Hb(FeII)NO could be achieved. Standard curves derived from synthetic preparations of SNO-Hb, Hb(FeII)NO, and S-nitrosoglutathione were linear (R>0.99), virtually superimposable, and revealing of sensitivity limits of approximately 1 nM. Two analytical criteria were then found to reliably distinguish SNO-Hb from Hb(FeII)NO: 1) signals from SNO-Hb were eliminated by pretreatment of samples with 10-fold excess $HgCl_2$, while Hb(FeII)NO was resistant to mercury challenge; and 2) treatment of SNO-Hb with $HgCl_2$ produced nitrite (by standard Griess reactions) in quantitative yields, whereas similar treatment of Hb(FeII)NO did not. UV/VIS spectroscopy confirmed that NO remained attached to heme in the presence of excess $HgCl_2$.

Example 9: Inhibition of Platelet Aggregation by S-Nitrosohemoglobins

Methods to prepare human HbA_0 were as described in Example 1 "Methods" section. Methods to make SNO-Hb(FeII)O_2 were as described for Example 2A. Methods to make SNO-Hb(FeIII) were as in Example 1 (see parts B, C, and "Methods" in Example 1). Quantifications of SNO-

hemglobins were made as in Example 1 according to the method of Saville (Saville, B., *Analyst 83*:670-672 (1958) and by the assay as described in Example 8, "Method for assay of S-nitrosohemoglobin."

Venous blood, anticoagulated with 3.4 nM sodium citrate, was obtained from volunteers who had not consumed acetylsalicylic acid or any other platelet-active agent for at least 10 days. Platelet-rich plasma was prepared by centrifugation at 150 x g for 10 minutes at 25°C and was used within 2 hours of collection. Platelet counts were determined with a Coulter counter (model ZM) to be 1.5 to 3 x 10^8/ml.

Aggregation of platelet-rich plasma was monitored by a standard nephelometric technique in which results have been shown to correlate with bleeding times. Aliquots (0.3 ml) of platelets were incubated at 37°C and stirred at 1000 rpm in a PAP-4 aggregometer (Biodata, Hatsboro, PA). Hemoglobins were preincubated with platelets for 10 min and aggregations were induced with 5 μM ADP. Aggregations were quantified by measuring the maximal rate and extent of change of light transmittance and are expressed as a normalized value relative to control aggregations performed in the absence of hemoglobin.

The results of the aggregation assays are shown in Figures 7A, 7B and 7C. Standard deviations are shown as vertical bars. SNO-Hb[Fe(II)]O_2 causes some inhibition of platelet aggregation at the higher concentrations tested. SNO-Hb[Fe(III)] also inhibits platelet aggregation when present at concentrations of 1 μM and above, but to a much greater extent than SNO-Hb[Fe(II)]O_2.

Example 10: Effect of SNO-Hbs on cGMP

Platelet rich plasma (PRP) was incubated with either hemoglobin, SNO-oxy Hb, or SNO-metHb for 5 min, after which the assay was terminated by the addition of 0.5 ml of ice

cold trichloroacetic acid to 10%. Ether extractions of the supernatant were performed to remove trichloroacetic acid, and acetylation of samples with acetic anhydride was used to increase the sensitivity of the assay. Measurements of cyclic GMP were performed by radioimmunoassay (Stamler, J. *et al., Circ. Res. 65*:789-795 (1989).

Results are shown in Figure 8. For all concentrations of Hb tested (1, 10 and 100 μM), the concentration of cGMP measured for SNO-Hb(FeIII) was less than that of native Hb.

Example 11: Polynitrosation of Hb

A. HbA_0 (oxy) was incubated with S-nitrosoglutathione at a ratio of 6.25 S-nitrosoglutathione/HbA_0 for 240 minutes at pH 7.4 at 25°C and desalted over Sephadex G-25 columns. Spectra were run in the presence (spectrum B, Figure 9A) and absence (spectrum A, Figure 9A) of dithionite. The shift in the spectrum is indicative of 2 SNO groups/tetramer.

B. HbA_0 was incubated with 100-fold excess S-nitrosoglutathione over protein for 240 minutes at pH 9.2, followed by desalting over a G-25 column. A portion was then treated with dithionite. The spectra in Figure 9B indicate that Hb has been nitrosated at multiple sites.

C. HbA_0 was treated with 100-fold excess S-nitroscysteine over tetramer at pH 7.4, 25°C for 5-20 min. After various times of treatment, the protein was desalted over a G-25 column and treated with dithionite. The spectra show progressive polynitrosation of Hb with time (spectra A to F in Figure 9C). After 5 minutes of treatment with 100-fold excess S-nitrosocysteine, 0.09 NO groups had added per tetramer (spectrum A of Figure 9C); after 20 minutes, at least 4 NO groups had added (spectrum F). At intermediate time points, 0.4 NO groups (spectrum B), 1.58 NOs (spectrum

C), 2.75 NOs (spectrum D) or 2.82 NOs had added per tetramer (spectrum E).

D. Rat Hb was treated with 100x S-nitrosoglutathione excess over tetramer for 3 hours at pH 7.4. The protein was then desalted by passage through a G-25 column. A portion of the desalted protein was treated with dithionite (spectrum B in Figure 9D; the protein of spectrum A was left untreated by dithionite). Spectrum B in Figure 9D is illustrative of a ratio of 6 RNOs/Hb.

E. A time course experiment tracking the extent of nitrosation of HbA_0 with time was performed (Figure 9E). Treatment of HbA_0 was with 10x excess S-nitrosocysteine at pH 7.4, 25°C or with 100x excess S-nitroscysteine under the same conditions. Analysis for SNO and NO was performed by the method of Saville and by UV spectroscopy as in Jia, L. *et al.*, *Nature 380*:221-226 (1996). Under these conditions the heme is ultimately oxidized; the rate is time dependent.

Treatment with 10x excess S-nitrosocysteine nitrosylates only the thiol groups of the two reactive cysteine residues of HbA_0. Inositol hexaphosphate is known to shift the allosteric equilibrium towards the T-structure (ordinarily, the deoxy form). Treatment with 100x excess nitrosates additional groups; i.e., the product has more than 2 NO groups/tetramer.

Example 12: Effect of SNO-Hb(FeII)O_2 on Blood Flow

SNO-Hb(FeII)O_2, having a SNO/Hb ratio of 2, was prepared (from HbA_0) by reaction with S-nitrosothiol. Rats breathing 21% O_2 were injected (time 0) with Hbs prepared from HbA_0 as indicated in Figure 10 (open circles, SNO-Hb (100 nmol/kg); filled circles, SNO-Hb (1000 nmol/kg); filled squares, unmodified Hb (1000 nmol/kg)). Three rats

were used per experiment. Blood flow was measured in brain using the H_2 clearance method; microelectrodes were placed in the brain stereotactically. Concomitant PO_2 measurements revealed tissue PO_2 = 20. Thus, SNO-Hb improves blood flow to the brain under normal physiological conditions, whereas native Hb decreases blood flow. NO group release is promoted by local tissue hypoxia.

Example 13: Effects of SNO-Hb(FeII)O_2, SNO-Hb(FeIII) and $(NO)_x$-Hb(FeIII) on Tension of Rabbit Aorta

Hemoglobin was treated with either 1:1, 10:1 or 100:1 S-nitrosocysteine to Hb tetramer for 1 hour, processed as in Example 4. The products of the reactions done with 1:1 and 10:1 excess were assayed by the Saville assay and by standard spectrophotometric methods. The product of the reaction done at the 1:1 ratio is SNO-Hb(Fe)O_2; SNO-Hb(FeIII) is found following reaction with 1:10 excess CYSNO/tetramer.

The aortic ring bioassay was performed as described in Example 4. The product of the reaction in which a ratio of 100:1 CYSNO/Hb tetramer was used, contains 2 SNOs as well as NO attached to the heme. The potency of the 100:1 CYSNO/Hb product is much greater than that of SNO-Hb(FeIII) and is indicative of polynitrosylation (see Figure 11).

Table 2

Endogenous levels of S-nitrosohemoglobin and nitrosyl(FeII)-hemoglobin in blood

Site	SNO-Hb (nM)	Hb(FeII)NO (nM)
Arterial	311 ± 55*	536 ± 99 †
Venous	32 ± 14	894 ± 126

* $P < 0.05$ vs venous; † $P < 0.05$ for paired samples vs venous

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

What is claimed is:

1. A method for delivering NO to the cells in a mammal, comprising administering low molecular weight nitrosating agent to the mammal.

2. A method for increasing the O_2-delivery capacity of hemoglobin in a mammal, comprising administering a low molecular weight NO donating agent to the mammal.

3. A method for scavenging oxygen free radicals in a mammal, comprising administering low molecular weight nitrosating agent to the mammal.

4. A method for preserving a living organ *ex vivo*, comprising perfusing the organ with a composition comprising nitrosated hemoglobin and low molecular weight thiol or NO donating agent.

5. A method for treating a blood borne disease in a patient, comprising the steps of:
   a) isolating the patient's red blood cells;
   b) treating the patient's red blood cells with S-nitrosothiol; and
   c) readministering to the patient the red blood cells.

6. The method of Claim 5, wherein the blood borne disease is malaria.

7. A method for treating a disease or medical disorder in a mammal, comprising administering to the mammal a nitrosating agent.

8. The method of Claim 7 in which the nitrosating agent is selected for rapid entry into the target cell.

9. The method of Claim 7, wherein the disease or medical disorder is selected from the group consisting of: shock, angina, stroke, reperfusion injury, acute lung injury, sickle cell anemia and infection of red blood cells.

10. A composition comprising SNO-Hb[FeII]O_2 which is S-nitrosylated without detectable oxidation of the heme Fe.

11. A method for making SNO-Hb[FeII]O_2, which is specifically S-nitrosylated on thiol groups, comprising incubating excess nitrosating agent with purified hemoglobin in the presence of oxygen.

12. The method of Claim 11 in which the nitrosating agent is a low molecular weight S-nitrosothiol.

13. A composition comprising of SNO-Hb[FeII] which is S-nitrosylated without detectable oxidation of the heme Fe.

14. A method for making SNO-Hb[FeII], which is specifically S-nitrosylated on thiol groups, comprising incubating excess nitrosating agent with purified hemoglobin in the absence of oxygen.

15. The method of Claim 14 in which the nitrosating agent is a low molecular weight S-nitrosothiol.

16. A method for regulating delivery of oxygen and NO, in various redox forms, in a mammal, comprising

administering to the mammal a mixture of a low molecular weight thiol or nitrosothiol and hemoglobin or nitrosated hemoglobin, selected for the oxidation state of the heme iron and for the oxygenation state.

17. A method for delivering NO in a mammal, comprising administering to the mammal a blood substitute comprising nitrosated hemoglobin.

18. The method of Claim 17, in which the blood substitute comprises nitrosated hemoglobin and low molecular weight S-nitrosothiol.

19. A method for scavenging oxygen free radicals and NO· in a mammal, comprising administering to the mammal a blood substitute comprising nitrosated hemoglobin.

20. A method for reducing blood pressure in a mammal, comprising administering nitrosated hemoglobin to the mammal.

21. A method for treating a disease in a mammal, comprising administering a form of nitrosated or nitrated hemoglobin to the mammal, wherein the disease is selected from the group consisting of heart disease, brain disease, vascular disease, atherosclerosis, lung disease and inflammation.

22. A method for treating a medical condition in a mammal, comprising administering a form of nitrosated hemoglobin to the mammal, wherein the medical condition is selected from the group consisting of stroke, angina and acute respiratory distress.

23. A method for enhancing the preservation of an excised organ, comprising storing the organ in a solution comprising SNO-Hb(FeII)O_2.

24. A method for treating a human with sickle cell anemia comprising administering to the human a preparation comprising SNO-Hb(FeII)O_2.

25. The method of Claim 24 in which the preparation comprises SNO-Hb(FeII)O_2 and a thiol.

26. The method of Claim 24 in which the preparation comprises SNO-Hb(FeII)O_2 and an S-nitrosothiol.

27. A method for treating a patient having a disease or medical condition characterized by abnormalities of nitric oxide and oxygen metabolism, comprising administering to the patient an effective amount of a preparation comprising nitrosated hemoglobin.

28. The method of Claim 27 in which the disease or medical condition is selected from the group consisting of: heart disease, lung disease, sickle-cell anemia, stroke, sepsis or organ transplantation.

29. A blood substitute comprising nitrosated or nitrated hemoglobin.

30. A method for treating a disorder resulting from platelet activation or adherence in an animal or human, comprising administering a composition comprising nitrosated or nitrated hemoglobin in a therapeutically effective amount.

31. The method of Claim 30 wherein the disorder is selected from the group consisting of: myocardial infarction, pulmonary thromboembolism, cerebral thromboembolism, thrombophlebitis, sepsis and unstable angina.

32. A method for preventing thrombus formation in an animal or human, comprising administering a composition comprising nitrosated hemoglobin in a therapeutically effective amount.

33. A method for regulating platelet activation in an animal or human, comprising administering, in a therapeutically effective amount, a composition comprising a substance which controls the allosteric equilibrium or spin state of hemoglobin.

34. The method of Claim 33 in which the substance converts the allosteric state of hemoglobin from R-structure to T-structure.

35. A method for forming polynitrosated hemoglobin, comprising combining hemoglobin with an excess of S-nitrosothiol over hemoglobin tetramer in an aqueous solution, and maintaining the resulting combination under conditions appropriate for nitrosation to occur at multiple sites on hemoglobin.

36. A method for forming polynitrosated or polynitrated hemoglobin in which heme Fe is in the FeII state, comprising combining hemoglobin with an NO donating compound, maintaining the resulting combination under conditions appropriate for nitrosation or nitration to occur, thereby forming polynitrosated or polynitrated hemoglobin, and reacting the polynitrosated or

polynitrated hemoglobin with a reagent which selectively reduces FeIII to FeII.

37. The method of Claim 36 in which the reagent which selectively reduces FeIII to FeII is a cyanoborhydride.

38. The method of Claim 36 in which the reagent which selectively reduces FeIII to FeII is methemoglobin reductase.

39. A composition comprising polynitrosated hemoglobin.

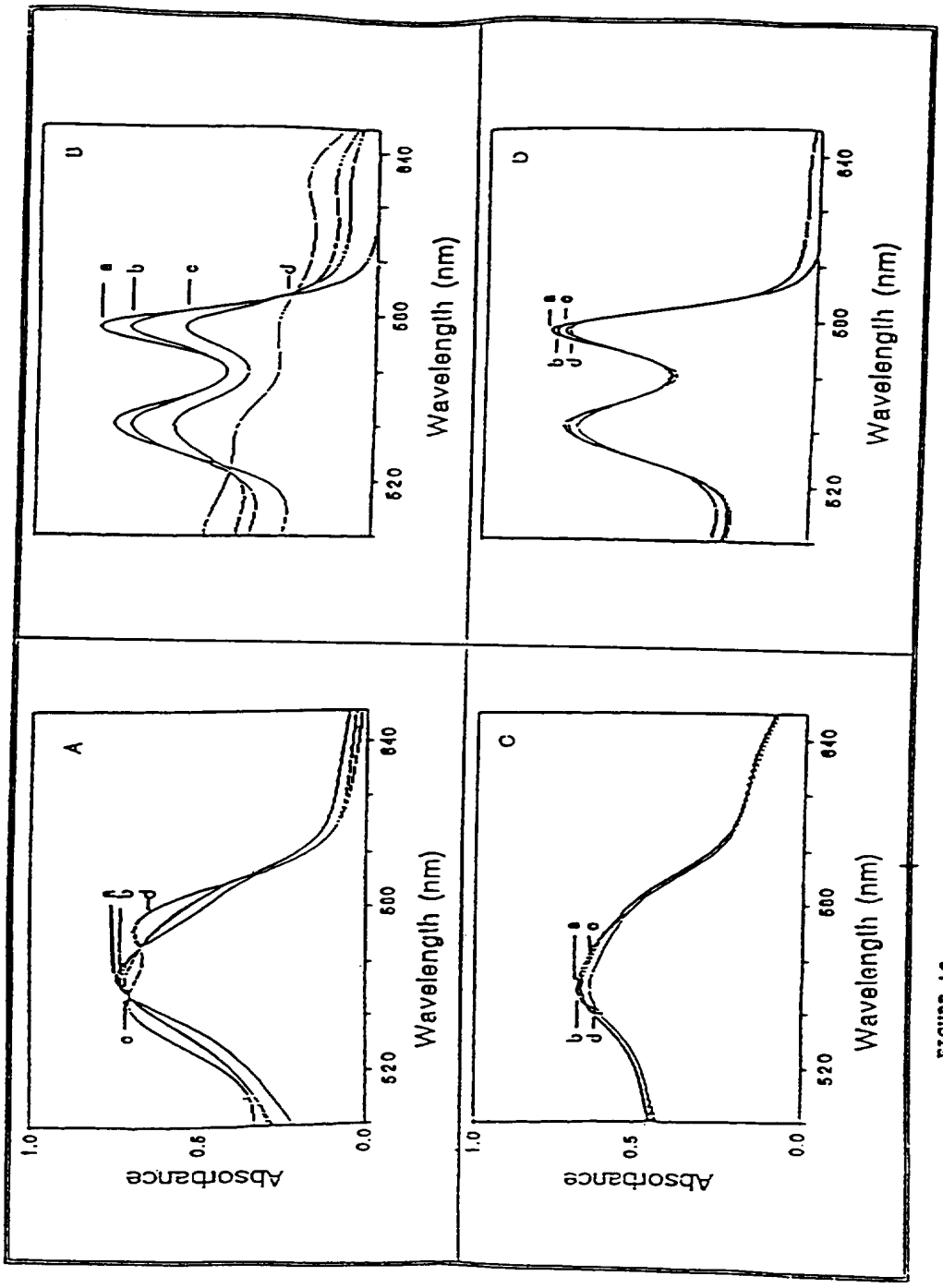

FIGURE 1A

FIGURE 1B

FIGURE 1C

FIGURE 1D

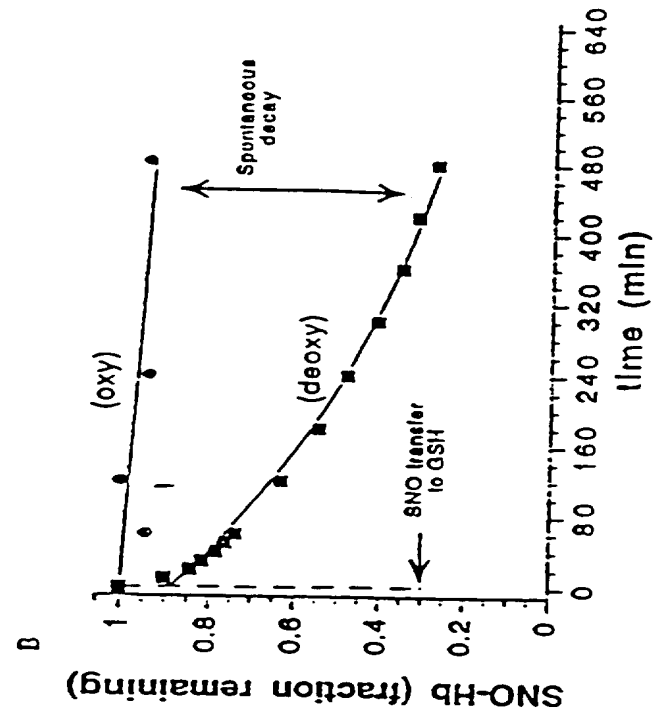

FIGURE 2B

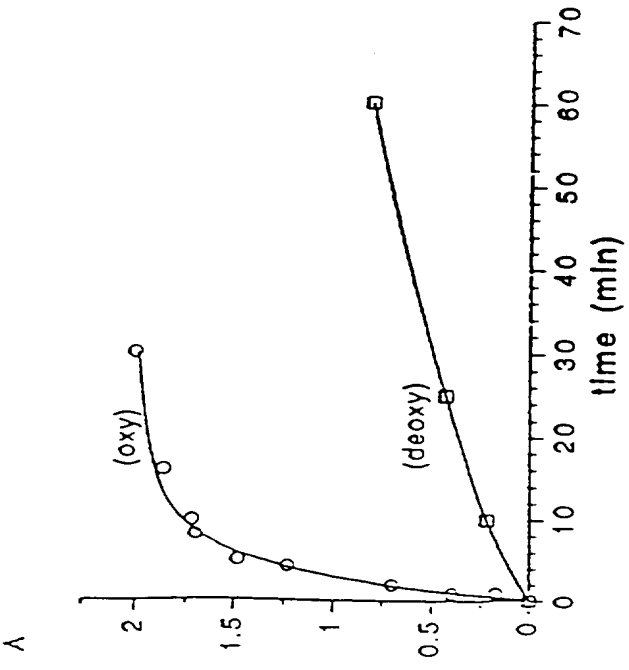

FIGURE 2A

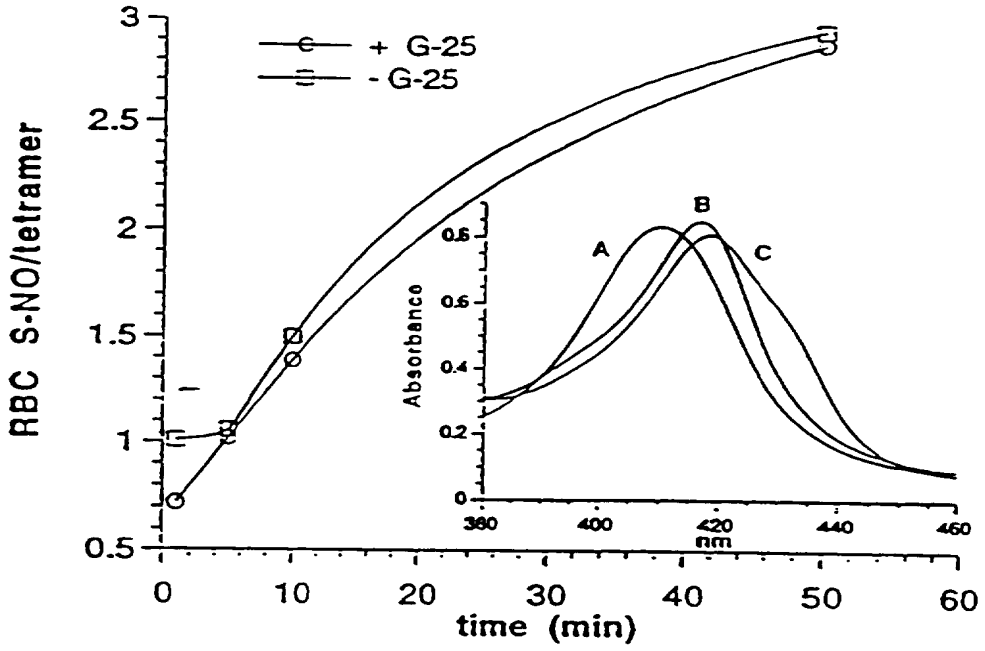

FIGURE 3A

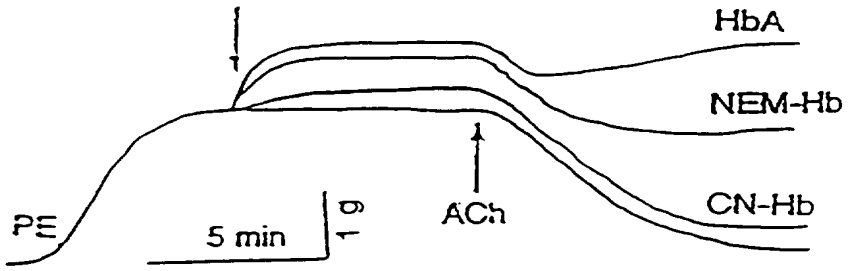

FIGURE 3B

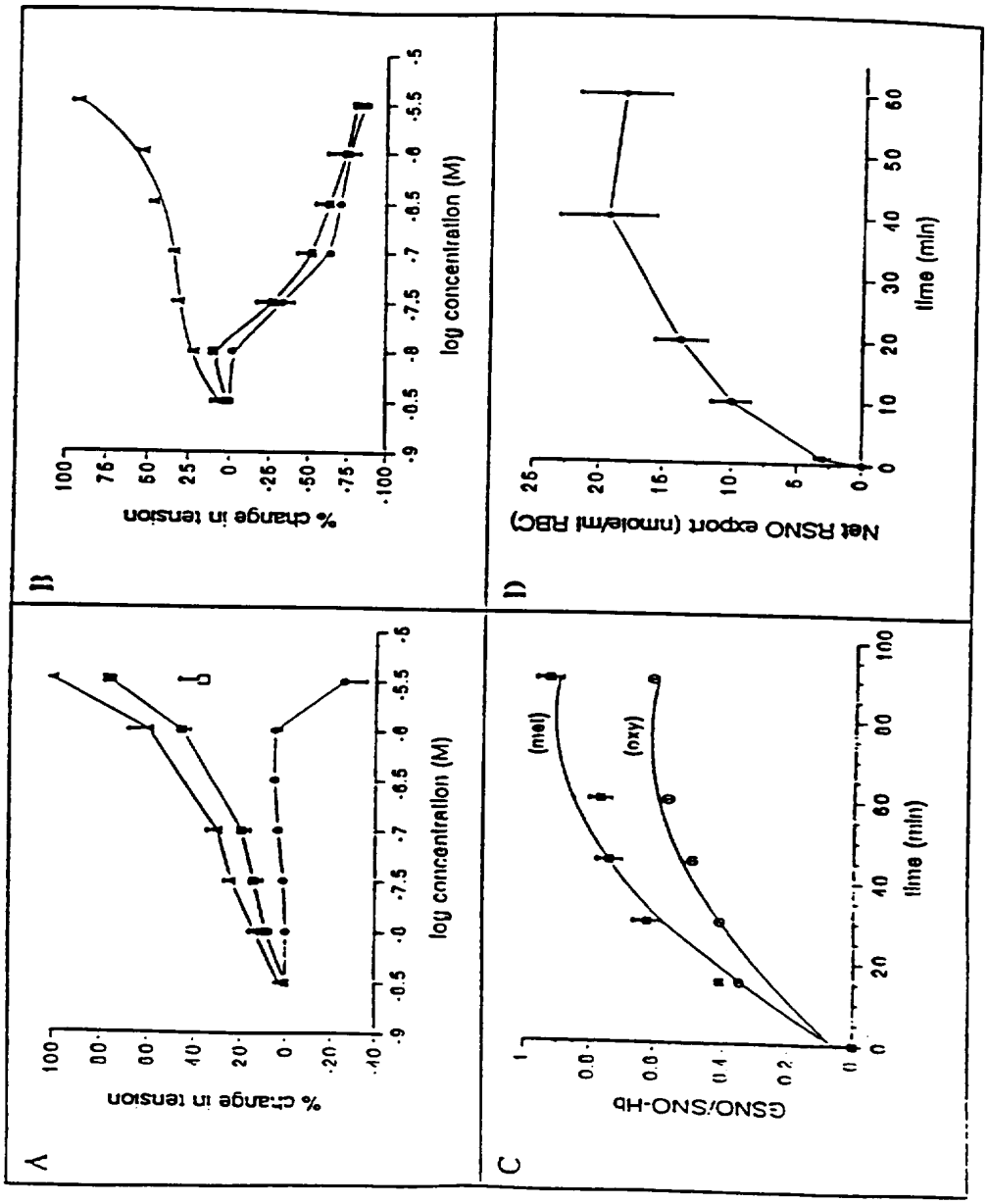

FIGURE 4A

FIGURE 4B

FIGURE 4C

FIGURE 4D

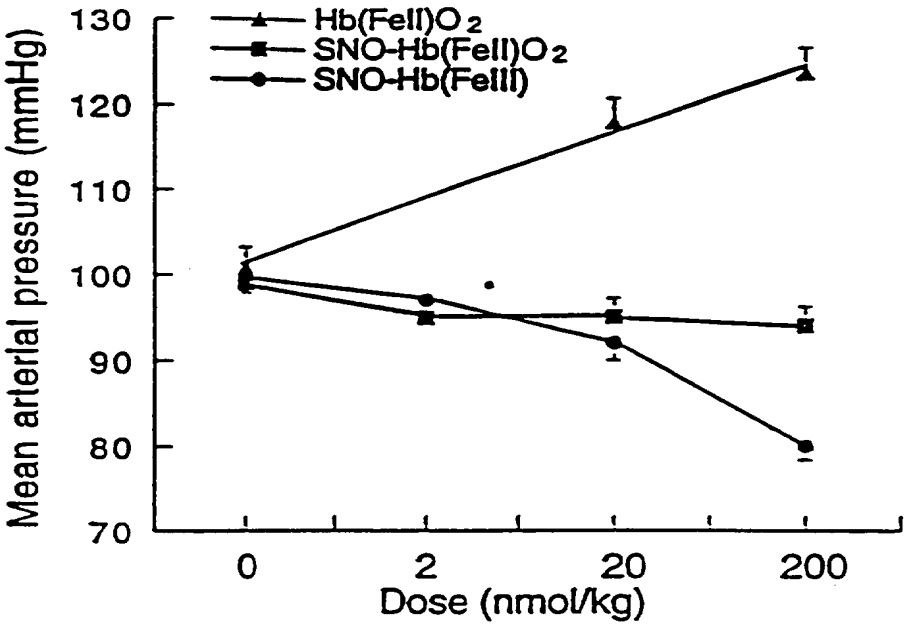
Hb(FeII)O2
SNO-Hb(FeII)O2
SNO-Hb(FeIII)
Mean arterial pressure (mmHg)
130
120
110
100
90
80
70
0
2
20
200
Dose (nmol/kg)

FIGURE 5

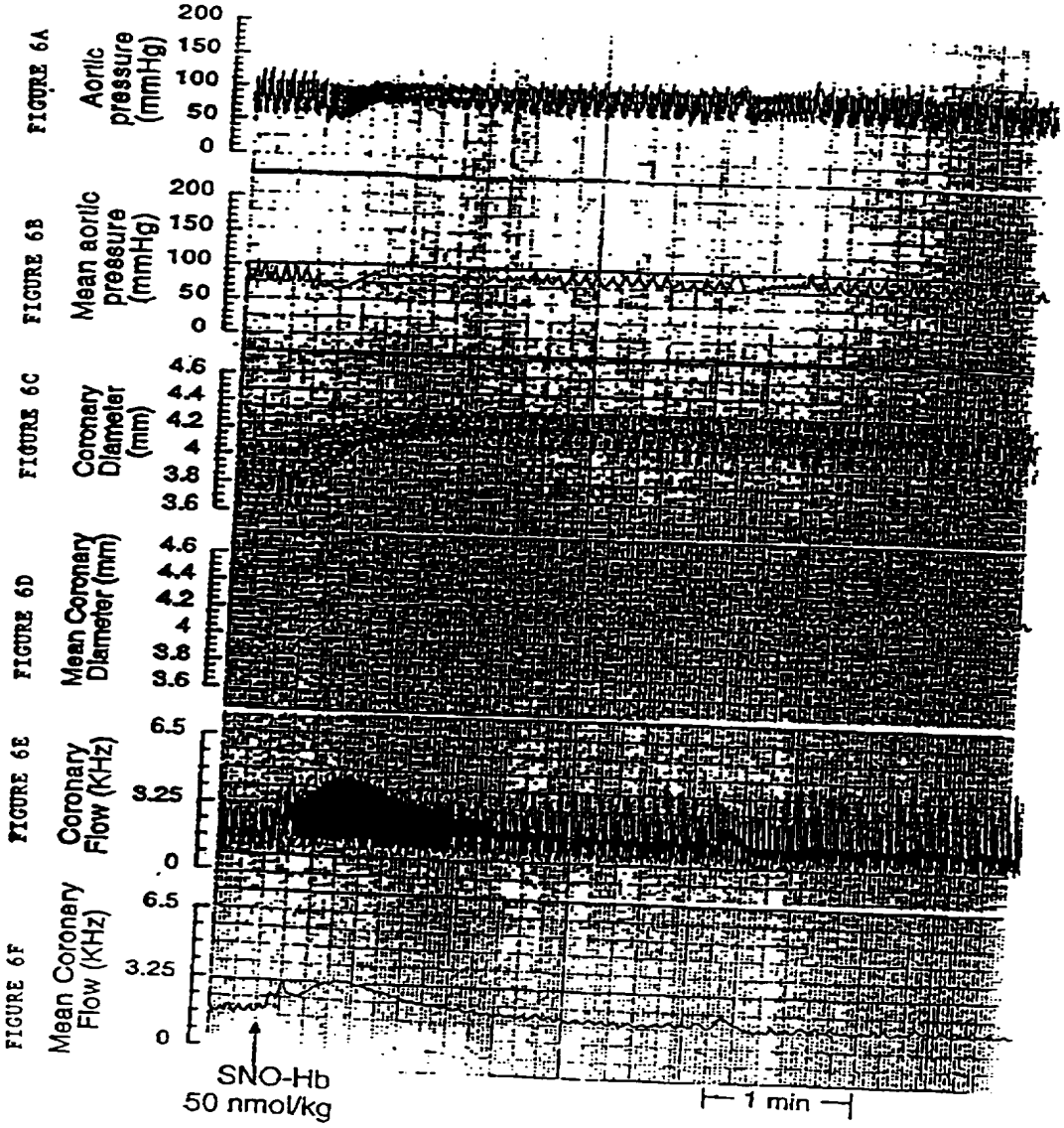
FIGURE 6A
Aortic pressure (mmHg)
200
150
100
50
0
FIGURE 6B
Mean aortic pressure (mmHg)
200
150
100
50
0
FIGURE 6C
Coronary Diameter (mm)
4.6
4.4
4.2
4
3.8
3.6
FIGURE 6D
Mean Coronary Diameter (mm)
4.6
4.4
4.2
4
3.8
3.6
FIGURE 6E
Coronary Flow (KHz)
6.5
3.25
0
FIGURE 6F
Mean Coronary Flow (KHz)
6.5
3.25
0
SNO-Hb
50 nmol/kg
1 min

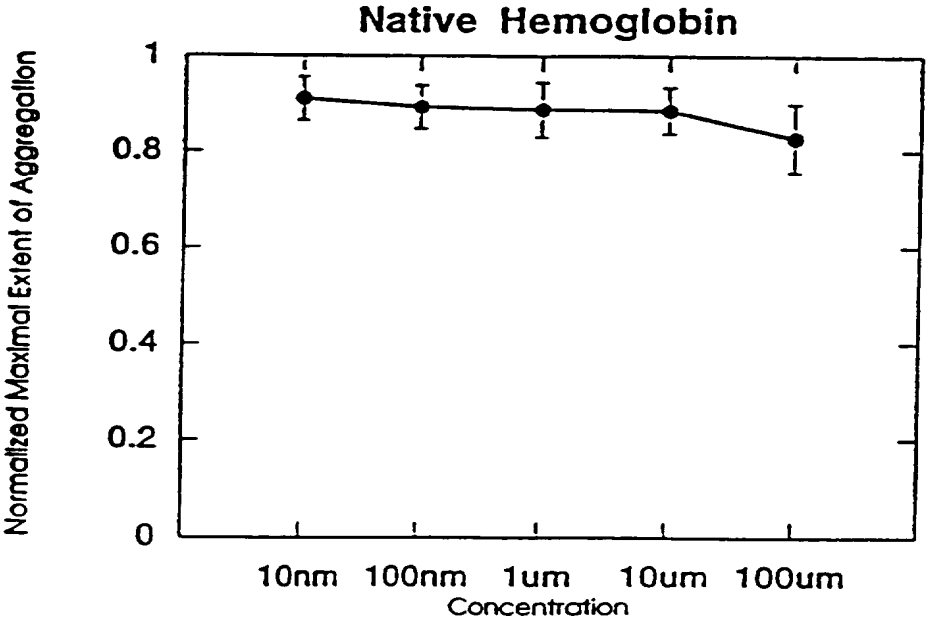
Native Hemoglobin
Normalized Maximal Extent of Aggregation
1
0.8
0.6
0.4
0.2
0
10nm
100nm
1um
10um
100um
Concentration

FIGURE 7A

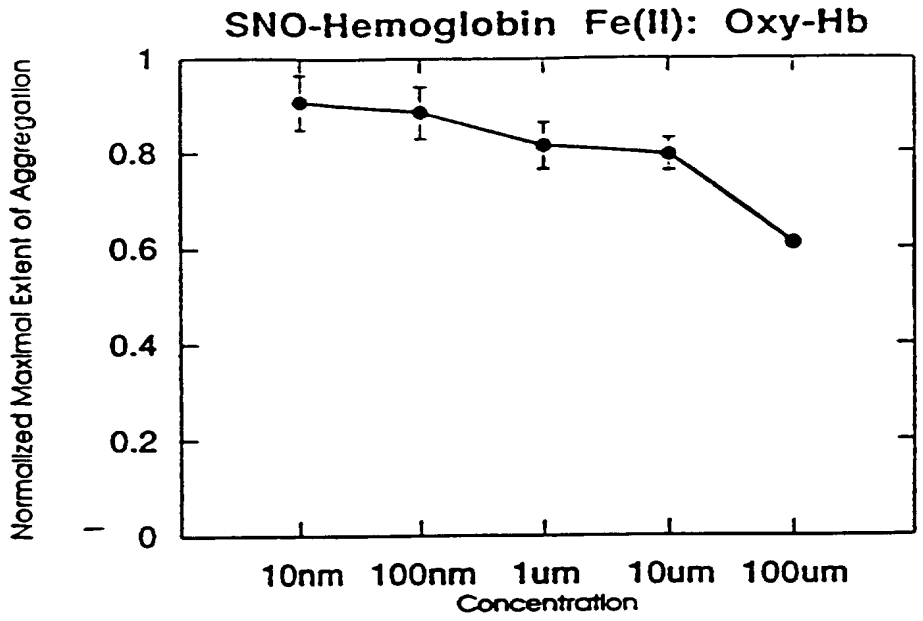

FIGURE 7B

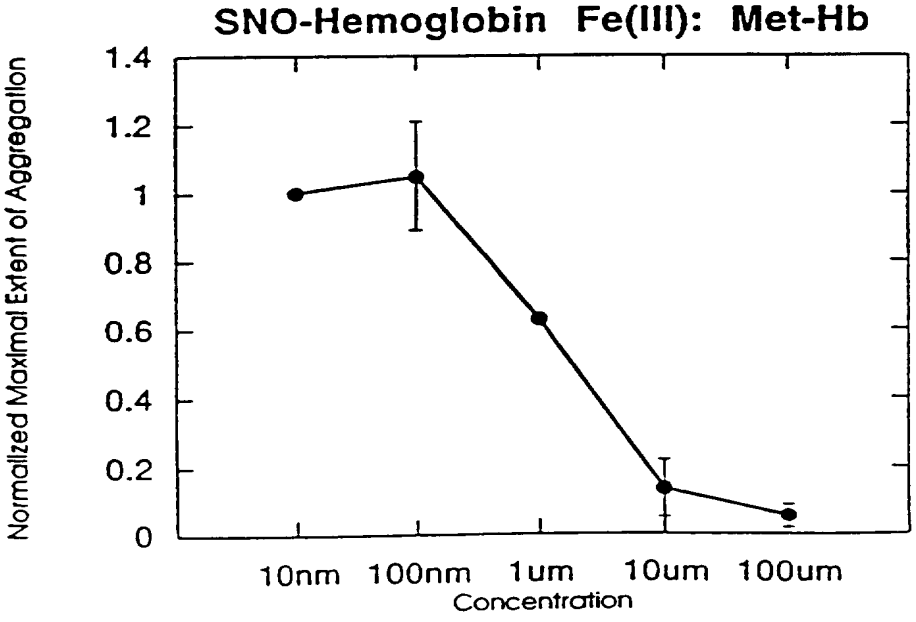

FIGURE 7C

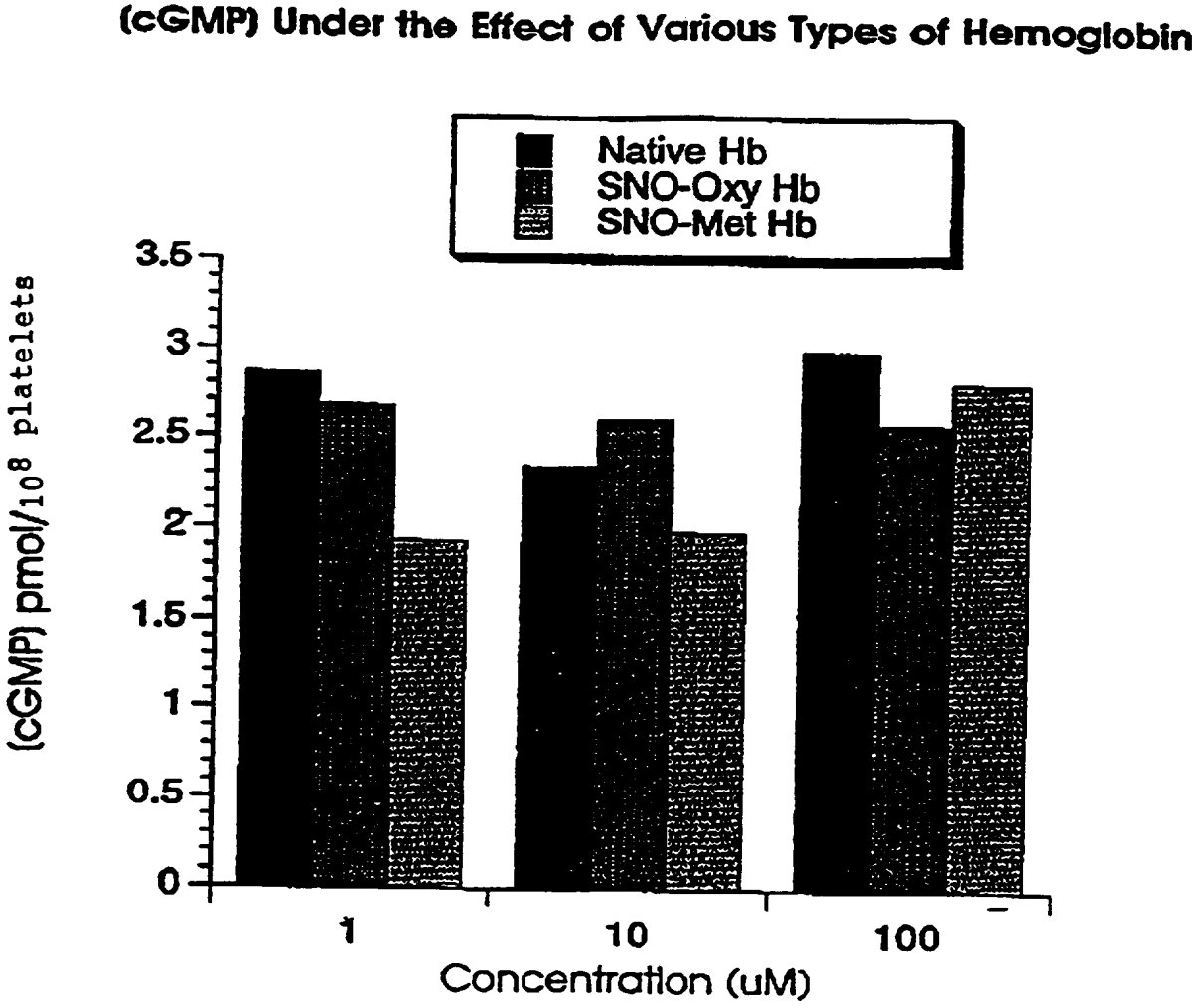
(cGMP) Under the Effect of Various Types of Hemoglobin
Native Hb
SNO-Oxy Hb
SNO-Met Hb
(cGMP) pmol/10^8 platelets
3.5
3
2.5
2
1.5
1
0.5
0
1
10
100
Concentration (uM)

FIGURE 8

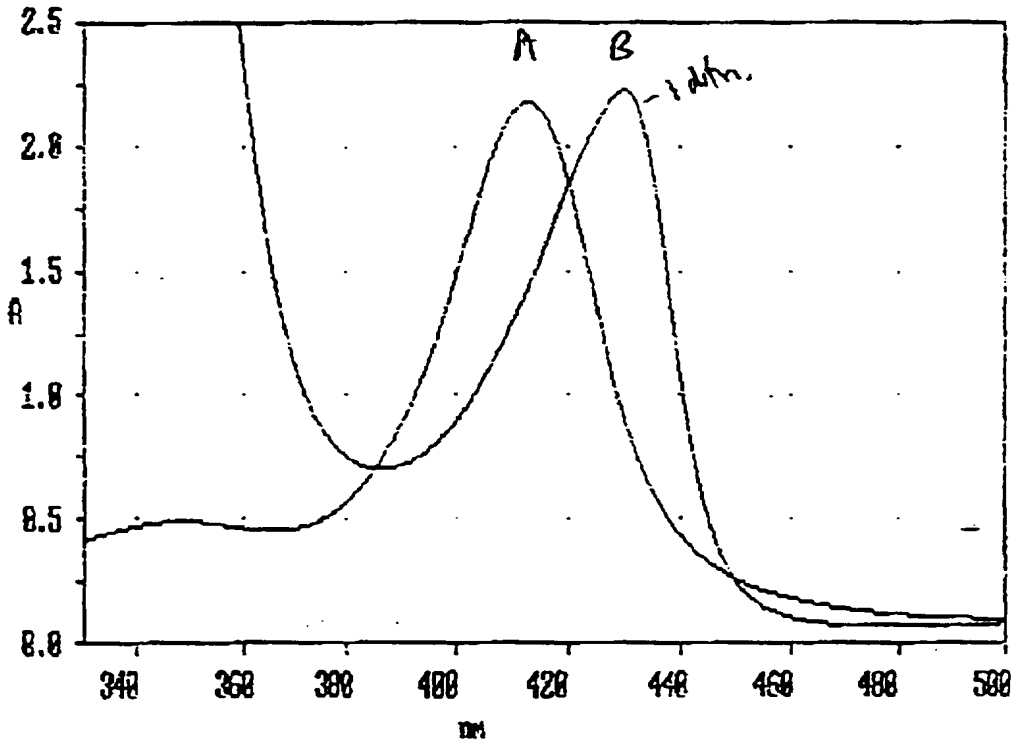

FIGURE 9A

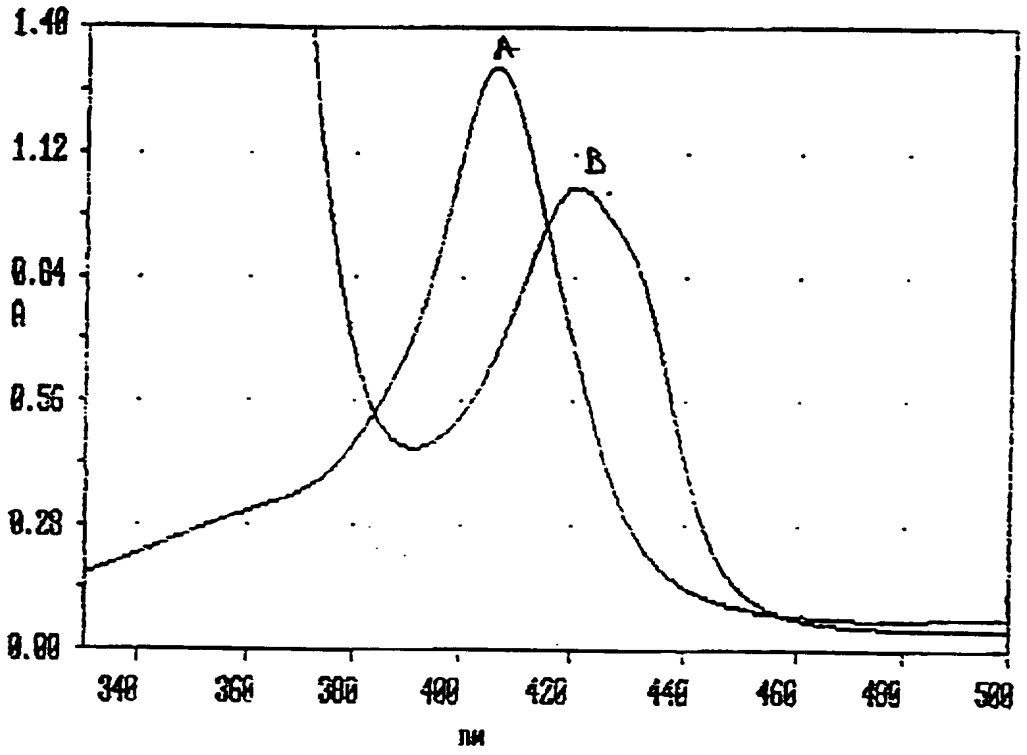

FIGURE 9B

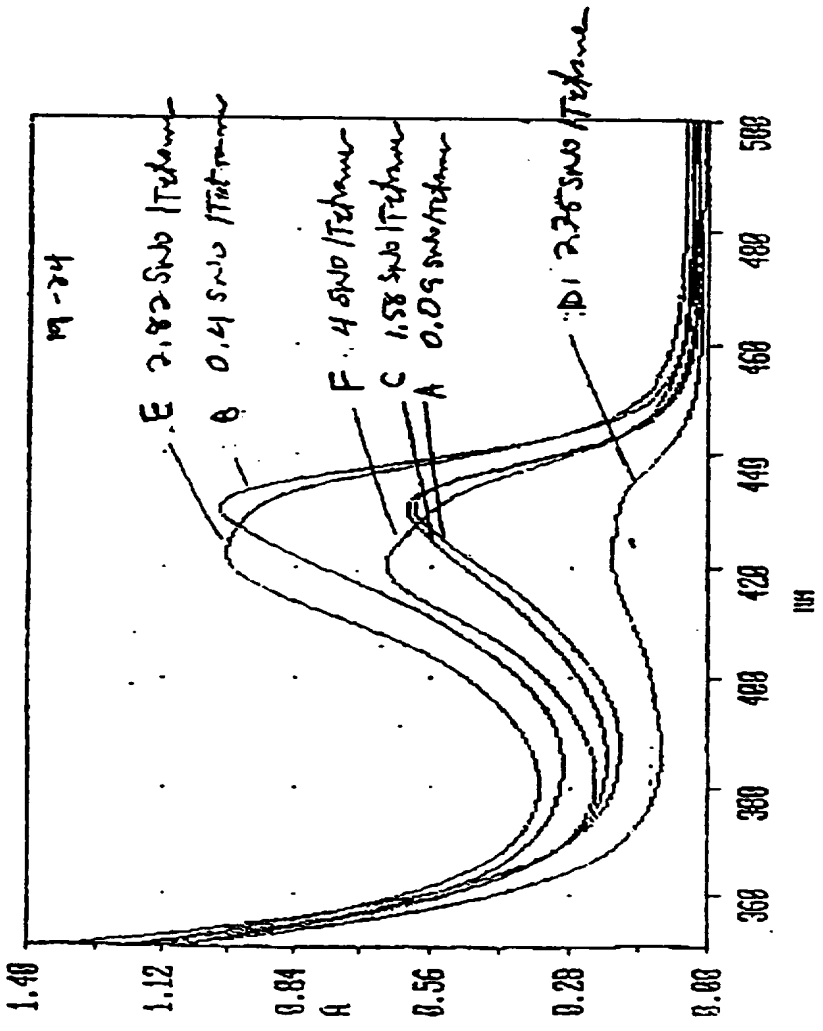
M-24
E 2,82 SMD ITEtham
B 0,41 SMD ITEtham
F 4 SMD ITEtham
C 1,58 SMD ITEtham
A 0,09 SMD ITEtham
D 2,35 SMD ITEtham
1.40
1.12
0.84
0.56
0.28
0.00
A
360
380
400
420
440
460
480
500
NM

FIGURE 9C

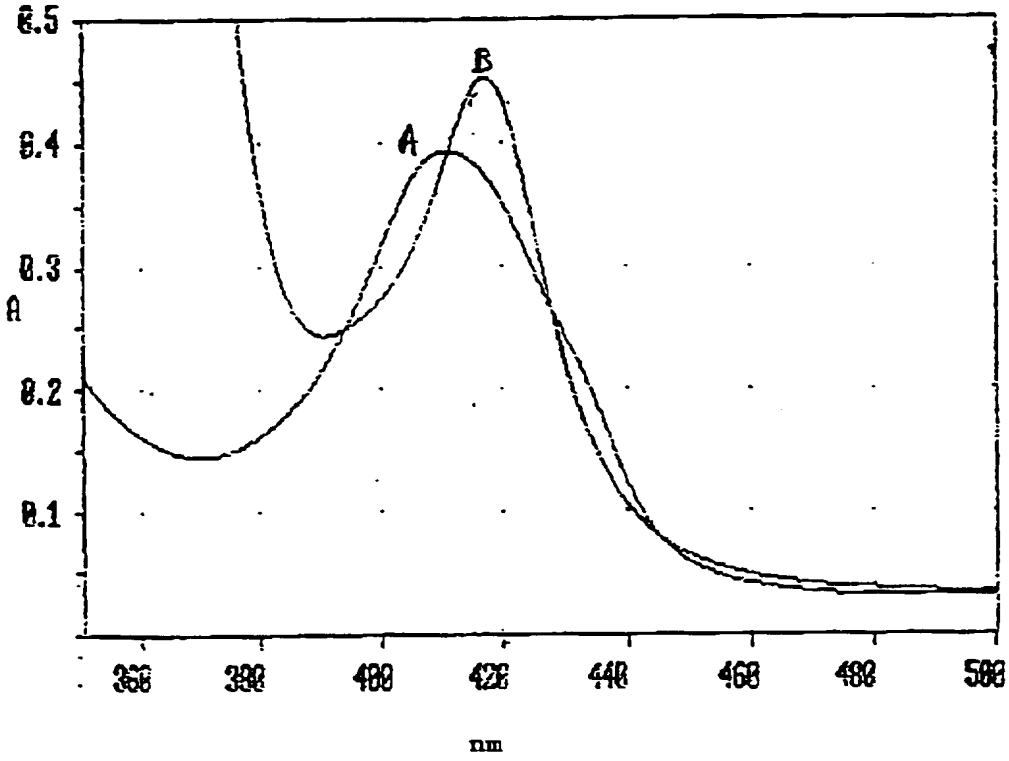

FIGURE 9D

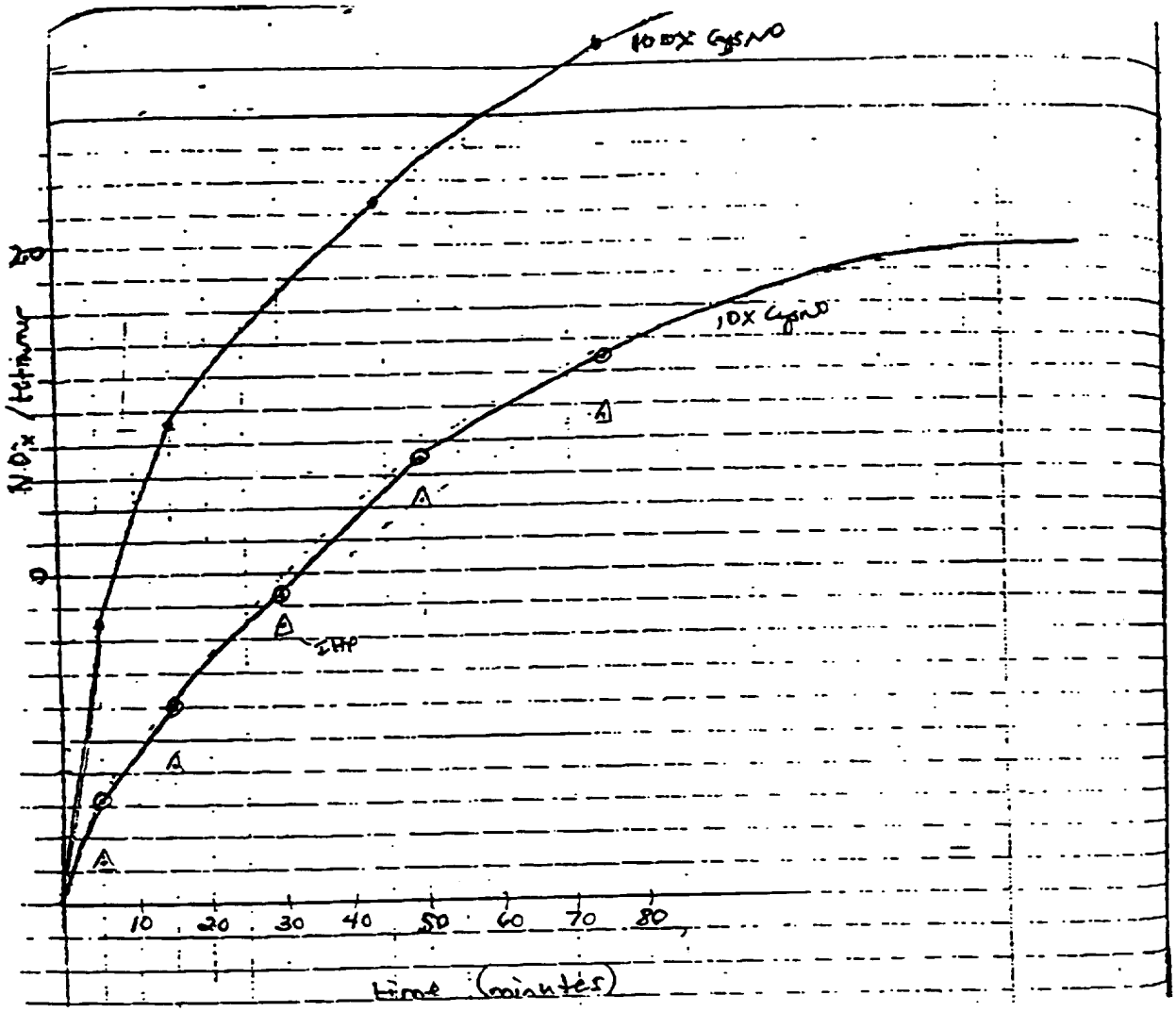

FIGURE 9E

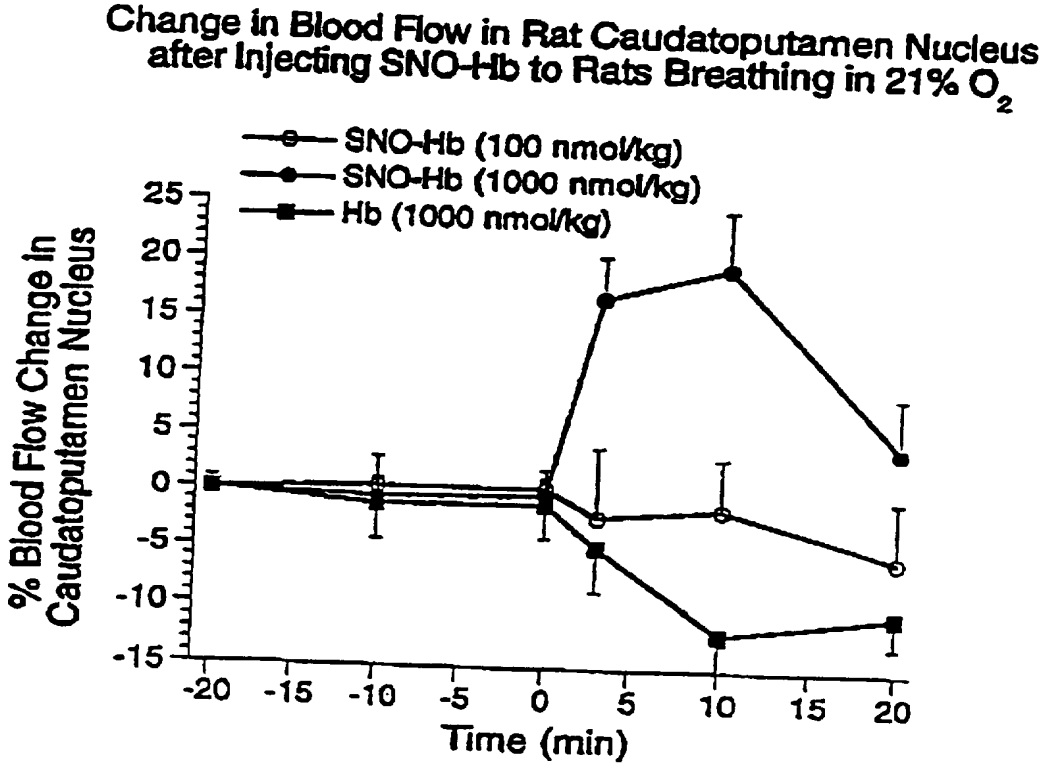
Change in Blood Flow in Rat Caudatoputamen Nucleus after Injecting SNO-Hb to Rats Breathing in 21% O_2
SNO-Hb (100 nmol/kg)
SNO-Hb (1000 nmol/kg)
Hb (1000 nmol/kg)
% Blood Flow Change in Caudatoputamen Nucleus
25
20
15
10
5
0
-5
-10
-15
-20
-15
-10
-5
0
5
10
15
20
Time (min)

FIGURE 10

COMPARISON OF EFFECTS OF HEMOGLOBIN AND S-NITROSYLATED HEMOGLOBIN WITH CYSNO ON TENSION OF RABBIT AORTAS

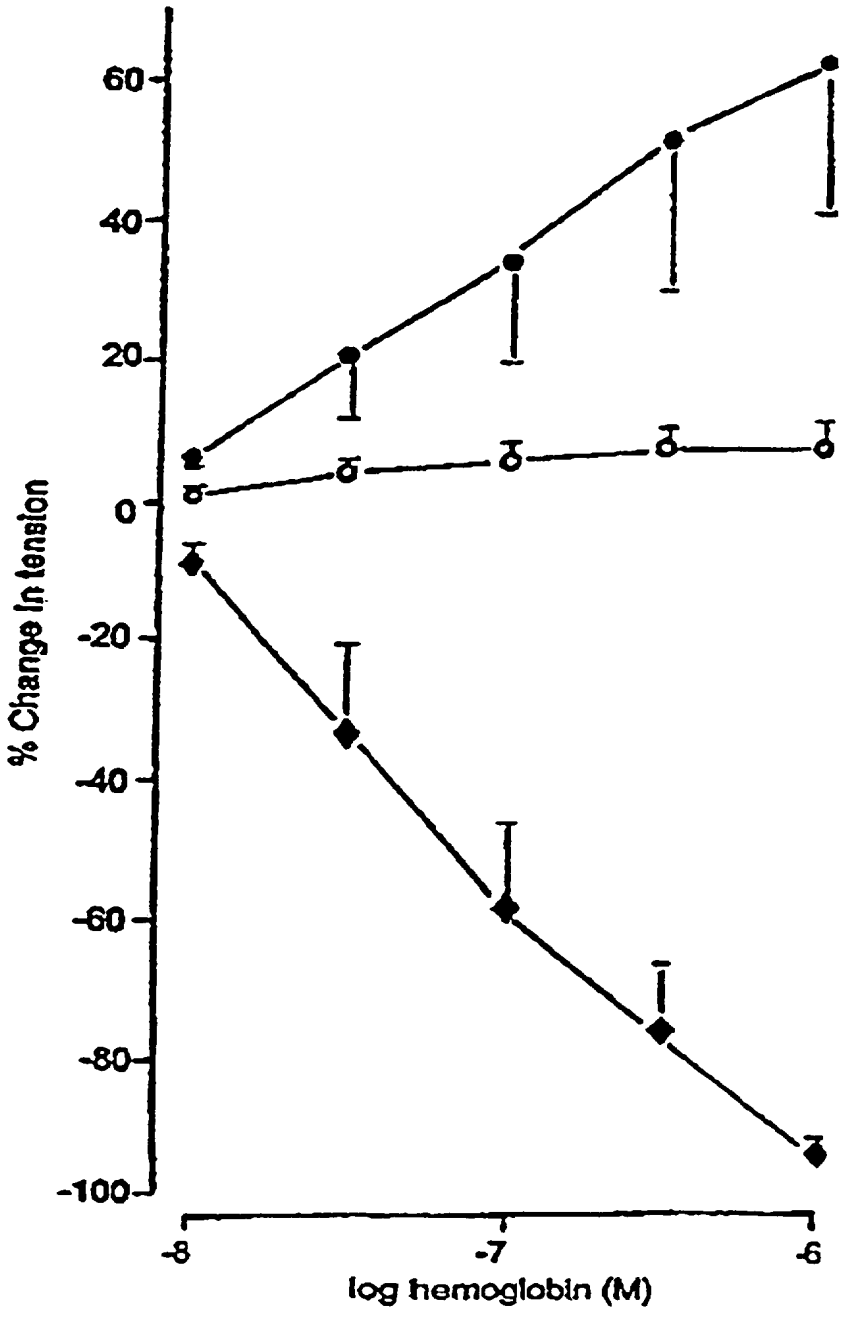

FIGURE 11

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 96/14659

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/00 A61K38/00 A61K31/04 A61K38/42 A61K31/095

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 09806 (BRIGHAM & WOMENS HOSPITAL) 27 May 1993 see the whole document ---	1,2,4,5, 7-9, 20-22, 30,32,33
X	WO,A,93 12068 (BRIGHAM & WOMENS HOSPITAL) 24 June 1993 see the whole document --- -/--	1,2,4,7, 8,10-14, 20-23, 28,30-34

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 1997	21.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	A. Jakobs

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/14659

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AM. J. PHYSIOL., vol. 266, no. 5, 1994, pages 1400-1405, XP000615617 KOSAKA, H. ET AL.: "ESR spectral transition by arteriovenous cycle in nitric oxide hemoglobin of citokine-treated rats" see the whole document ---	1,2,16
X	TOXICOL. APPL. PHARMACOL., vol. 94, no. 3, 1988, pages 458-465, XP002022225 KRUSZYNA, R. ET AL.: "Generation of Valency hybrids and Nitrosylated Species of Hemoglobin in Mice by Nitric Oxide Vasodilators" see the whole document ---	1,7,17, 20,36
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 259, no. 1, 1984, pages 80-87, XP002022226 DOYLE ET AL.: "Structural Effects in Alkyl Nitrite Oxidation of Human Hemoglobin" see the whole document ---	2,10-14
X	ARCHIVES OF SURGERY, vol. 129, no. 2, 1994, pages 158-164, XP000615627 SHAH, N.S. ET AL.: "Efficacy of Inhaled Nitric Oxide in a Porcine Model of Adult Respiratory Distress Syndrome" see the whole document ---	1,7-9
X	EUROPEAN HEART JOURNAL, vol. 12, no. Suppl. E, 1991, pages 16-24, XP000615639 KUKOVETZ, W.R. ET AL.: "Cellular mechanism of action of therapeutic nitric oxide donors" see the whole document ---	1,7-9
X	ARTIF. CELLS, BLOOD SUBSTITUTES, IMMOBILIZATION BIOTECHNOL., vol. 23, no. 3, 1995, pages 271-276, XP000615495 GREENBURG, A.G. ET AL.: "Nitrosyl Hemoglobin formation in-vivo after intravenous administration of a hemoglobin-based oxygen carrier in endotoxemic rats." see the whole document ---	1,7,9, 16,17

-/--

3

INTERNATIONAL SEARCH REPORT

In' tional Application No
PCT/US 96/14659

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANAL. BIOCHEM., vol. 191, no. 1, 1990, pages 138-143, XP000616299 CLANCY ET AL.: "Use of Thionitrobenzoic Acid to Characterize the Stability of Nitric Oxide in Aqueous Solutions and in Porcine Aortic Endothelial Cell Suspensions." see the whole document	3,33
A	BLOOD, vol. 47, no. 3, 1976, pages 481-488, XP000615579 CHARACHE, S. ET AL.: "Evaluation of Extracorporeal Alkylation of Red Cells as a Potential Treatment for Sickle Cell Anemia" see the whole document	28
A	BIOCHEM, BIOPHYS. RES. COMM., vol. 54, no. 3, 1973, pages 1024-1029, XP000615619 WHEELER, G.P. ET AL.: "Anti-sickling activity of nitrosoureas" see the whole document	1,7,9

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 96/ 14659

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 1-9,16-22,24-28,30-34 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 33-34
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

Claims 33 and 34 where searched as relating to nitrosated thiol compounds in order to avoid lack of unity objections.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found muluple inventuons in this international applicatuon, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the inventuon first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/US 96/14659

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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